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EFFECT OF SUCCINIC AND MALIC ACIDS AND FRUCTOSE ON KETOSIS IN ALLOXAN-DIABETIC RATS*

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The current concept of fatty acid metabolism involves the formation of acetyl CoA[1] and the condensation of acetyl CoA with oxalacetate upon entrance into the tricarboxylic acid cycle. Therefore, a sufficient amount of oxalacetic acid is necessary for the complete oxidation of fatty acids via the tricarboxylic acid cycle, and, if sufficient oxalacetic acid is not present, ketosis results.

Previously (1) we have demonstrated that the administration of various precursors of oxalacetic acid, such as succinic and malic acids, and oxalacetic acid itself, decreases total urinary ketone body excretion in non-diabetic rats made ketotic by the administration of butyric acid. This raises the question as to whether or not these substances would have the same effect in an insulin-deficient preparation. Glucose requires insulin to produce oxalacetate at a normal rate, but the effect of insulin on the rate of production of oxalacetate from other precursors is unknown, although recent work (2) indicates that insulin is involved in metabolism at the level of the tricarboxylic acid cycle. There are a few reports in the literature on the effects of succinic acid on ketosis in diabetic humans (3), but the results are conflicting. Therefore, we have investigated the effects of succinic and malic acids on ketosis in insulin-deficient rats. In view of the reports that the utilization of fructose is independent of insulin (4), the effects of fructose on ketosis in these diabetic animals were also investigated.

EXPERIMENTAL

Female Sprague-Dawley rats weighing 200 to 220 gm. were used throughout these experiments. The rats were made diabetic by the subcutaneous injection of 8 mg. per 100 gm. of recrystallized alloxan monohydrate (10 per cent solution). No rats were used less than 4 weeks after alloxan administration, because we have previously shown (5) that ketonuria may be severe directly after the administration of alloxan and some rats do not reach plateau levels of ketonuria in less than 4 weeks. The only rats found

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[1] Coenzyme A.

to be sufficiently diabetic and yet able to withstand the experiment and remain in good clinical condition were animals with non-fasting blood glucose levels of 450 to 550 mg. per cent. To get dependable ketotic preparations we fasted these diabetic rats 8 hours and at 4.30 p.m. administered 4 to 6 cc. of Wesson oil by stomach tube. The rats were then placed in metabolism cages, and the urine was collected under toluene for a first period of 16 hours. The next morning at 8.30 a.m. another dose of 4 to 6 cc. of Wesson oil was administered and the urine collected for the succeeding 24 hours (second collection period). 2 cc. of Amphojel and 0.2 cc. of a 1:10 dilution of tincture of opium were administered with the oil to prevent diarrhea. Only about 50 per cent of the animals survived the first experiment. However, the rats that proved suitable could usually be used for one experiment per week for several months. These selected animals remained in good clinical condition. While the degree of ketosis established in different rats by the above procedure varied, the level of ketosis for each rat was relatively constant.

Ketone bodies (expressed as acetone) were determined by the method of Greenberg and Lester (6), and glucose was estimated by Somogyi's modification of the Shaffer-Hartmann method (7). The ketone body and glucose excretions in the first urine collection period were determined, and, if either value was out of the control range established for this rat, the experiment was terminated, and the following week the entire experiment was started over again. In this way each rat served as a constant control upon itself. The test solutions were given in five divided doses at 8.30 a.m., 11.00 a.m., 2.00 p.m., 4.30 p.m., and 7.30 p.m. during the second collection period (24 hours). Succinate and malate were given after neutralization to pH 4.5 with NaOH. Therefore, in the control experiments the rats received amounts of Na^+, in NaCl or $NaHCO_3$ solution, equivalent to the maximal amount of Na^+ used. This served as a check on the possible effect of the Na^+ *per se* and on the production of alkalosis with sodium succinate or malate. The amounts of succinate and malate administered were equivalent (on the basis of possible production of oxalacetate) to a dose of 2 cc. per 100 gm. per 24 hours of a 13.5 per cent or a 20 per cent solution of glucose. Fructose was given as a 20 per cent solution (2 cc. per 100 gm. per 24 hours).

RESULTS AND DISCUSSION

In these experiments no significant difference was found in the ketonuria of rats given NaCl or $NaHCO_3$ solutions ($P > 0.10$ Table I). However, in contrast to the decrease in ketonuria produced by succinate and malate in non-diabetic rats (1), succinate and malate did not decrease ketonuria in these insulin-deficient animals, although the dose administered caused marked glucosuria (Tables II and III). This is in agreement with Bess-

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TABLE I

Comparison of Effect of $NaHCO_3$ and NaCl Solutions on Urinary Glucose and Ketone Body Excretion of Alloxan-Diabetic Rats Given Wesson Oil (Second Collection Period)

Ketone bodies reported in mg. per 100 gm. per 24 hours and glucose in gm. per 100 gm. per 24 hours.

NaCl		$NaHCO_3$	
Ketone bodies	Glucose	Ketone bodies	Glucose
22	0.09	26	0.13
41	0.21	49	0.23
28	0.21	23	0.16
34	0.16	39	0.18
25	0.05	17	0.16
53	0.14	49	0.23
7	0.13	12	0.14
49	0.15	49	0.24
41	0.32	43	0.28

TABLE II

Effect of Insulin, Succinate, and Succinate and Insulin on 24 Hour Urinary Excretion (Second Collection Period) of Glucose and Total Ketone Bodies in Insulin-Deficient Rats Given Wesson Oil

The amount of succinate given was equivalent (on the basis of oxalacetic acid potential) to a dose of 2 cc. per 100 gm. per 24 hours of a 13.5 per cent glucose solution. Ketone bodies reported in mg. per 100 gm. per 24 hours and glucose in gm. per 100 gm. per 24 hours.

Control		Succinate		Insulin		Succinate and insulin		Insulin dose, units
Ketone bodies	Glucose	Ketone bodies	Glucose	Ketone bodies	Glucose	Ketone bodies	Glucose	
49–43	0.10–0.17	52	0.40	14	0	2.0	0	1.0
27	0.30	23	0.43					
54–51	0.28–0.18	52	0.62	50	0	21	0.36	0.6
47–41	0.19–0.12	54	0.52	40	0.06	11	0.42	0.7
45–55	0.11–0.12	80*	0.78	49	0.08	20	0.41	0.7
25–23	0.12–0.11	32	0.37	4.0	0.04	0.9	0	1.0
44–45	0.19–0.17	45	0.48	22	0	2.2	0.09	0.8
36	0.16	37	0.40	28	0.04	14	0	0.6
55	0.19			52	0.12	0.7	0.10	0.8
34–42	0.10–0.12	77*	0.60	43	0.09	5.4	0.08	0.7
66	0.36	80*	0.76					
51	0.28	52*	0.62					

* The amount of succinate administered was equivalent, on the basis of oxalacetate potential, to a dose of 2 cc. per 100 gm. per 24 hours of a 20 per cent glucose solution.

man[2] who gave 5 mmoles of sodium succinate per kilo to human diabetics in coma and observed no change in blood ketones, although the blood sugar increased 90 mg. per cent within 1½ hours. Assuming a two-third distribution of sugar throughout the entire body water, he calculated that this rise would account for complete conversion of succinate to glucose following the usual path of 2 moles of succinate to 1 mole of glucose. In a few instances the administration of large doses of succinate or malate to

TABLE III

Effect of Insulin, Malate, and Insulin and Malate on 24 Hour Urinary Excretion (Second Collection Period) of Glucose and Total Ketone Bodies in Insulin-Deficient Rats Given Wesson Oil

The amount of malate given was equivalent (on the basis of oxalacetate potential) to a dose of 2 cc. per 100 gm. per 24 hours of a 13.5 per cent glucose solution. Ketone bodies reported in mg. per 100 gm. per 24 hours and glucose in gm. per 100 gm. per 24 hours.

Control		Malate		Insulin		Malate and insulin		Insulin dose, units
Ketone bodies	Glucose	Ketone bodies	Glucose	Ketone bodies	Glucose	Ketone bodies	Glucose	
39	0.18	140*	0.78	8	0.09	2	0.11	0.5
		70*	0.56					
22	0.34	22	0.32					
53	0.14	66*	0.40	25	0.07	16	0.35	0.7
61	0.24			42	0.08	4	0.12	0.7
58	0.32			40	0.09	12	0.18	0.6
31	0.18	24	0.52	30	0.09	16	0.12	0.6
26	0.22	44	0.38	20	0.01	12	0.14	0.4
42	0.17	40		40		20	0.33	0.6
43	0.31	43	0.58					

* Dose of malate equivalent, on the basis of oxalacetate potential, to a dose of 2 cc. per 100 gm. per 24 hours of a 20 per cent glucose solution.

our rats appeared to increase ketonuria. This could be explained on the basis of a direct conversion of some succinate to acetate (8) and condensation of 2 acetate molecules to acetoacetate. This ketotic effect of succinate or malate was not observed in non-diabetic ketotic preparations (1).

In order to investigate the rôle insulin may have in succinate metabolism, we determined a dose of insulin which caused no change or a small decrease in the ketonuria of our rats (Tables II and III). When this dose of insulin was administered in conjunction with succinate or malate, the alloxan-diabetic rats showed a much greater decrease in ketonuria than when only insulin was given.

[2] S. P. Bessman, personal communication.

n succinate per kilo to human diabetics blood ketones, although the blood sugar 1½ hours. Assuming a two-third dis- entire body water, he calculated that ete conversion of succinate to glucose, of succinate to 1 mole of glucose. In a large doses of succinate or malate to

BLE III

and Malate on 24 Hour Urinary Excretion Glucose and Total Ketone Bodies in Rats Given Wesson Oil

ivalent (on the basis of oxalacetate poten- 24 hours of a 13.5 per cent glucose solution. gm. per 24 hours and glucose in gm. per 100

Insulin		Malate and insulin		Insulin dose, units
Ketone bodies	Glucose	Ketone bodies	Glucose	
8	0.09	2	0.11	0.5
25	0.07	16	0.35	0.7
42	0.08	4	0.12	0.7
40	0.09	12	0.18	0.6
30	0.09	16	0.12	0.6
20	0.01	12	0.14	0.4
40		20	0.33	0.6

basis of oxalacetate potential, to a dose of 2 cent glucose solution.

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It has been demonstrated that the administration of succinate to humans with diabetes mellitus causes an increase in blood pyruvate concentrations (9) and blood glucose levels.[2] In our own experiments the administration of both malate and succinate caused an increase in glucosuria without affecting the ketonuria. The above evidence indicates that a significant proportion of these test substances entered the usual pathway of metabolism and ran back via oxalacetate through the glycolytic cycle. In six experiments the larger doses of succinate or malate appeared to cause an increase in ketone body excretion. A "ketolytic" effect of succinate and malate could therefore be masked at the lower dosage level. However, we feel that, according to present concepts, a better explanation of the absence of the expected drop in ketonuria may be that utilization of the oxalacetate to decrease ketonuria does not occur in the absence of insulin, suggesting an interference in the condensation of acetyl CoA and oxalacetate.

Since a large part of succinate and malate is converted to glucose, thereby providing extra glucose, it is difficult to distinguish between an insulin effect on glucose and one on condensation of acetyl CoA with oxalacetate. However, if our fundamental assumption is correct that condensation of oxalacetate with acetyl CoA is necessary to prevent ketosis, these experiments appear to indicate that the oxalacetate from succinate and malate is less efficient for condensation in the absence of insulin than in its presence. It has been demonstrated (1) in normal animals made ketotic with butyrate that glucose, succinate, and malate are equally effective, on the basis of oxalacetate potential, in reducing ketosis.

Recent interest in fructose metabolism in diabetes led us to make a comparison of the effects of glucose and fructose on ketonuria in insulin-deficient animals fed Wesson oil. Because some fructose may be converted to glucose by the gastrointestinal tract (10), and because Cori (11) has shown that the gastrointestinal tract absorbs glucose and fructose at different rates, animals were given the sugars intraperitoneally, subcutaneously, and by mouth. The route of injection did not change the effect, probably because the material was given in small doses (five divided doses).

The doses of glucose and fructose used were often larger, on the basis of oxalacetate potential, than the doses of succinate and malate. This was necessary in order to compare the antiketogenic effects of these two substances.

Fructose is removed from the blood stream of diabetics more rapidly than glucose, fructokinase activity being independent of insulin (4). However, this fructose is then metabolized through anaerobic glycolysis. If there is a block in insulin-deficient preparations at the level of the tricarboxylic acid cycle, fructose should prove no more effective than glucose in decreasing ketosis, unless the concentration of fructose is increased suffi-

ciently to exert a mass effect following phosphorylation. Under the conditions of our experiments no difference could be detected between the effects of glucose and fructose on ketonuria (Table IV). Whittlesey and Zubrod (12) observed that fructose was a better ketolytic agent than glu

TABLE IV

Effect of Glucose and Fructose on 24 Hour Urinary Excretion of Glucose and Total Ketone Bodies in Insulin-Deficient Rats Given Wesson Oil

Ketone bodies reported in mg. per 100 gm. per 24 hours and glucose in gm. per 100 gm. per 24 hours.

Control		Glucose*		Fructose*		Route of administration
Ketone bodies	Glucose	Ketone bodies	Glucose	Ketone bodies	Glucose	
51–66	0.28–0.36	35	0.56	71	0.50	Oral
25	0.15	14	0.33	8	0.43	"
55	0.11	4	0.52	17	0.52	"
25–23	0.12–0.11	2	0.48	2	0.48	"
31	0.14	21†	0.71	18†	0.64	Intraperitoneal
42		18†	0.70	14†	0.73	Oral
34	0.09	16‡	0.37	16‡	0.36	Subcutaneous
51	0.18	29	0.58	29	0.86	"
34–33	0.16–0.18	9	0.39	11	0.32	"
38	0.09	3‡	0.29	5‡	0.30	"
34	0.09	3	0.43	2	0.39	"

* 2 cc. per 100 gm. per 24 hours of a 20 per cent solution.
† Repeat determinations on the same rat.
‡ 2 cc. per 100 gm. per 24 hours of a 13.5 per cent solution.

TABLE V

Effect of Fluoroacetate (0.25 Mg. per 100 Gm.) on 24 Hour Urinary Excretion (Second Collection Period) of Glucose and Total Ketone Bodies in Insulin-Deficient Rats Given Wesson Oil

Ketone bodies reported in mg. per 100 gm. per 24 hours and glucose in gm. per 100 gm. per 24 hours.

Control		Fluoroacetate		Control	
Ketone bodies	Glucose	Ketone bodies	Glucose	Ketone bodies	Glucose
3.1	0.05	35	0.30	1.7	0.14
1.7	0.10	31	0.40	2.1	0.12
2.6	0.18	30	0.18		
2.2	0.03	18	0.20	1.7	
4.3	0.12	8.2	0.25		
1.0	0.07	6.0	0.15		

llowing phosphorylation. Under the con-
ifference could be detected between the
n ketonuria (Table IV). Whittlesey and
se was a better ketolytic agent than glu-

ABLE IV

Hour Urinary Excretion of Glucose and Total
-Deficient Rats Given Wesson Oil

100 gm. per 24 hours and glucose in gm. per

e*	Fructose*		Route of administration
Glucose	Ketone bodies	Glucose	
0.56	71	0.50	Oral
0.33	8	0.43	"
0.52	17	0.52	"
0.48	2	0.48	"
0.71	18†	0.64	Intraperitoneal
	14†	0.73	Oral
	16‡	0.36	Subcutaneous
.58	29	0.86	"
.39	11	0.32	"
.29	5‡	0.30	"
.43	2	0.39	"

per cent solution.
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per cent solution.

E V

n.) *on 24 Hour Urinary Excretion (Second*
Total Ketone Bodies in Insulin-
ven Wesson Oil

n. per 24 hours and glucose in gm. per 100

etate	Control	
Glucose	Ketone bodies	Glucose
0.30	1.7	0.14
0.40	2.1	0.12
0.18		
0.20	1.7	
0.25		
0.15		

cose in depancreatized dogs. However, they administered their test substances intravenously, used a much larger dose than in these experiments, and observed their animals for short periods.

We are unable to explain adequately why these larger doses of glucose and fructose decreased ketonuria, while increasing the dose of succinate or malate to a comparable level (on the basis of oxalacetate potential) did not cause a drop in urinary ketone bodies in the absence of insulin.

Previously (1) we demonstrated that sodium fluoroacetate increased urinary ketone body excretion in non-diabetic rats fed butyric acid from 7.4 mg. per 100 gm. per 24 hours to 11.4 mg. per 100 gm. per 24 hours. When the same dose of sodium fluoroacetate was given to alloxan-diabetic rats, the ketonuria increased as much as 18 times (Table V). Fluoroacetate has been shown to block the tricarboxylic acid cycle at the citric acid stage (13).

SUMMARY

1. The administration of succinic and malic acids had no detectable effect on the ketonuria of alloxan-diabetic rats fed Wesson oil, although the dose was sufficient to produce an increased glucosuria.

2. The administration of small amounts of insulin plus succinic or malic acid caused a larger decrease in ketonuria, in our insulin-deficient preparation, than when insulin alone was given.

3. No difference was found between the effects of glucose and fructose on ketonuria in diabetic ketotic rats.

4. The tricarboxylic acid cycle inhibitor, fluoroacetate, has a greater effect in insulin-deficient preparations than in control rats.

5. These results are regarded as presumptive evidence that insulin may be involved in metabolism at the level of the tricarboxylic acid cycle, possibly in the condensation of acetyl CoA with oxalacetate.

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OBSERVATIONS ON THE EFFECT OF CHEMICAL CONFIGURATION ON THE EDEMA-PRODUCING POTENCY OF ACIDS, ALDEHYDES, KETONES AND ALCOHOLS[1, 2]

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In an earlier publication (Finnegan *et al.*, 1947) we described a method for quantitatively evaluating the edema-producing potency on mucous membranes, of the irritants in tobacco smoke. Subsequently we have adapted this method to permit of its use in evaluating the edema-producing potencies of chemicals in water solution. The method and results obtained with four classes of chemical compounds are detailed below.

METHOD. Morphinized male albino rabbits of about 2 kgm. weight are used. One-half hour after morphinization (20 mgm. per kgm. body weight subcutaneously) the rabbit is tied in a supine position to an animal board. A pocket is made of the upper lid of the right eye and filled with the solution containing the irritant. The solution is held in place for 3 minutes, after which the lid is released and the rabbit returned to the upright position. One hour after application of the irritant the rabbit is sacrificed by cerebral concussion, the upper palpebral conjunctiva of each eye excised, weighed, dried in an oven to constant weight, and reweighed. From the wet and dry weights the ratio of moisture to dry weight for each membrane is calculated. The difference between the ratio for the right (exposed) eye tissue and that for the left (control) eye tissue represents the degree of edema production.

In the present study 5 rabbits were used at each molar concentration tested and 6 such groups in studying each irritant, except in a few instances where solubility was a limiting factor. With each irritant, the concentrations tested were so adjusted (solubility permitting) as to produce average moisture to dry weight gains (right eye ratio minus left eye ratio) spreading over a range up to about 5. Analysis of regression was then applied to the data to determine the equation for the best line to fit all points on the resulting curve. In this calculation the average moisture to dry weight gains were used as such, but the molar concentrations of the test material were converted to log units. From the resulting equation, we have for comparative purposes arbitrarily calculated the molar concentration that should produce a 2.5 unit increase in moisture to dry weight. The 2.5 point represents about a 50 per cent gain in moisture to dry weight as compared to the control tissue.

RESULTS AND DISCUSSION. *Acids.* The results obtained on the acids studied are summarized in table 1.

Among the monobasic organic acids, excepting formic, there is a tendency toward increasing edema-producing potency (EPP) with increasing molecular weight. Unsaturation increased EPP in the case of acrylic acid (compare with propionic) but not in the case of crotonic acid (compare with butyric).

The dibasic acid, succinic, has a lower EPP than the corresponding monobasic

[1] A preliminary report appeared in Fed. Proc., 8: 312, 1949.

[2] This investigation has been supported under a research grant from the American Tobacco Company.

TABLE 1

Comparative edema-producing potency of certain acids

Acids	Molar Concentration to Produce 2.5 Unit Increase in Moisture to Dry Weight Ratio ± S.E.	Equation Relating Edema-Producing Potency to Molar Concentration*
Monobasic, sat.		
Formic	0.08 + 0.01	Y = 11.04 + 7.74 log X
Acetic	0.13 ± 0.02	Y = 10.94 + 9.42 log X
Propionic	0.12 ± 0.01	Y = 11.96 + 10.43 log X
Butyric	0.10 ± 0.01	Y = 11.37 + 8.86 log X
Valeric	0.086 ± 0.008	Y = 16.00 + 12.66 log X
Caproic	0.045 ± 0.005	Y = 20.15 + 13.14 log X
Monobasic, unsat.		
Acrylic	0.054 ± 0.01	Y = 12.26 + 7.69 log X
Crotonic	0.10 ± 0.01	Y = 13.90 + 11.41 log X
Dibasic, sat.		
Succinic	0.51 ± 0.07	Y = 6.67 + 14.21 log X
Dibasic, unsat.		
Maleic	0.23 ± 0.02	Y = 5.91 + 5.29 log X
Monobasic, hydroxy		
Lactic	0.35 ± 0.11	Y = 5.35 + 6.20 log X
Dibasic, hydroxy		
Malic	0.49 ± 0.03	Y = 6.26 + 12.28 log X
Dibasic, dihydroxy		
Tartaric	0.32 ± 0.02	Y = 7.19 + 9.59 log X
Inorganic		
Hydrochloric	0.12 ± 0.05	Y = 6.52 + 4.35 log X
Phosphoric	0.41 ± 0.22	Y = 4.58 + 5.39 log X

* Y = magnitude of increase in moisture to dry weight ratio; X = molar concentration of irritant.

acid, butyric. Among dibasic acids, unsaturation increased EPP in the one case tested (maleic vs. succinic acid).

Addition of a single hydroxyl group to the acid molecule decreased EPP in the case of the monobasic acid, lactic (compare with propionic), but not in the case of the dibasic acid, malic (compare with succinic), while addition of two hydroxyl groups increased EPP in the case of the dibasic acid, tartaric (compare with succinic).

The strongly ionized inorganic acid, hydrochloric, has no greater EPP than the more weakly ionized monobasic organic acids.

Aldehydes. The results obtained on the aldehydes studied are summarized in table 2.

Among the saturated aldehydes, with the possible exception of formaldehyde, increasing molecular weight results in increasing EPP. Unsaturation markedly increases the EPP of aldehydes (compare acrolein with propionaldehyde and crotonaldehyde with butyraldehyde), but in this case the EPP decreases with increasing molecular weight.

of certain acids

Equation Relating Edema-Producing Potency to Molar Concentration*
Y = 11.04 + 7.74 log X
Y = 10.94 + 9.42 log X
Y = 11.96 + 10.43 log X
Y = 11.37 + 8.86 log X
Y = 16.00 + 12.66 log X
Y = 20.15 + 13.14 log X
Y = 12.26 + 7.69 log X
Y = 13.90 + 11.41 log X
Y = 6.67 + 14.21 log X
Y = 5.91 + 5.29 log X
Y = 5.35 + 6.20 log X
Y = 6.26 + 12.28 log X
Y = 7.19 + 9.59 log X
Y = 6.52 + 4.35 log X
Y = 4.58 + 5.39 log X

ght ratio; X = molar concentra-

ncreased EPP in the one case

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EPP. Unsaturation markedly
n with propionaldehyde and
case the EPP decreases with

TABLE 2

Comparative edema-producing potency of certain aldehydes and ketones

Aldehydes	Molar Concentration to Produce 2.5 Unit Increase in Moisture to Dry Weight Ratio ± S.E.	Equation Relating Edema-Producing Potency to Molar Concentration*
Saturated		
Formaldehyde	0.84 ± 0.63	Y = 2.70 + 2.67 log X
Acetaldehyde	0.99 ± 0.14	Y = 2.53 + 8.78 log X
Propionaldehyde	0.60 ± 0.18	Y = 4.59 + 9.57 log X
Butyraldehyde	0.39 ± 0.04	Y = 5.44 + 7.11 log X
Unsaturated		
Acrolein	0.0017 ± 0.0006	Y = 21.74 + 6.94 log X
Crotonaldehyde	0.014 ± 0.002	Y = 14.91 + 6.73 log X
Ketones		
Saturated		
Acetone	3.9 ± 0.2	Y = −2.42 + 8.40 log X
Methyl-ethyl	1.8 ± 0.2	Y = −0.42 + 12.08 log X
Diethyl	Too insoluble	

* See footnote to table 1.

TABLE 3

Comparative edema-producing potency of certain alcohols

Alcohols	Molar Concentration to Produce 2.5 Unit Increase in Moisture to Dry Weight Ratio ± S.E.	Equation Relating Edema-Producing Potency to Molar Concentration*
Primary, sat.		
Methyl	7.9 ± 0.7	Y = −7.70 + 11.35 log X
Ethyl	4.8 ± 0.4	Y = −4.76 + 10.66 log X
Propyl	1.5 ± 0.2	Y = −0.24 + 15.39 log X
n-Butyl	0.58 ± 0.06	Y = 6.48 + 16.58 log X
Amyl	0.17 ± 0.01	Y = 19.36 + 22.21 log X
Hexyl	Too insoluble	
Primary, unsat.		
Allyl	2.5 ± 0.3	Y = 5.99 + 8.95 log X
Primary, iso.		
iso-Butyl	0.59 ± 0.03	Y = 6.67 + 18.27 log X
Secondary		
sec-Butyl	0.85 ± 0.10	Y = 3.25 + 17.74 log X
Tertiary		
tert-Butyl	1.8 ± 0.3	Y = −1.21 + 15.27 log X

* See footnote to table 1.

Ketones. The results obtained on ketones are also summarized in table 2. Lack of solubility prevented the testing of more than two members of this class but it would appear that EPP increases with increasing molecular weight.

Alcohols. The results obtained on alcohols are summarized in table 3.

Among primary alcohols, EPP increases markedly with increasing molecular weight, decreases with unsaturation (compare allyl with propyl), and appears to be unaffected by isomerism (compare iso-butyl with n-butyl).

In passing from primary to secondary to tertiary alcohols the EPP progressively declines (compare primary, secondary and tertiary butyl).

SUMMARY

A method for measuring the edema-producing potency of chemicals in water solution has been described. The results of its application to a study of the effect of chemical configuration on the edema-producing potency of acids, aldehydes, ketones and alcohols are summarized.

ACKNOWLEDGMENT. We are indebted to Miss Elizabeth Thompson and Miss Jacqueline Curtis for technical assistance in performing the reported experiments.

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Research Section

The Metabolism of L- and DL-Malic Acids by Rats

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(*Received 15 February 1969*)

Abstract—When L- or DL-malic acid, labelled with carbon-14, was administered orally or intraperitoneally to rats, each was extensively metabolized, 90–95% of the radioactivity being excreted within 24 hr, mainly in the expired air as [^{14}C]carbon dioxide (83–92% of the administered radioactivity). The acids were, moreover, metabolized at the same rate, which was found to be independent of the route of administration. There would thus appear to be no justification for discriminating against the use of D-malic acid as a food additive.

INTRODUCTION

The Joint FAO/WHO Expert Committee on Food Additives (1967) has made recommendations relating to the use of malic acid as an acidulant and flavouring agent. Malic acid exists in nature as the L-isomer, being present in apples and many other fruits and plants. Moreover, it is an intermediary metabolite in the aerobic utilization of pyruvate through the citric-acid cycle. The Committee proposed that while no limit should be set for the acceptable daily intake of L-malic acid by man, the daily intake of the D-isomer should not exceed 100 mg/kg body weight. In support of these proposals the Committee stated that whereas the metabolism of L-malic acid was well understood, little was known about the fate of the D-enantiomorph. In reviewing the biochemical aspects of malic acid metabolism, however, the Committee did not mention several studies relating to the oxidation of D-2-hydroxyacids, including D-malic acid.

Huennekens, Mahler & Nordmann (1951) demonstrated that D-malic acid could be oxidized by a crude suspension of mitochondria obtained from rabbit liver and kidney. Baker (1952) found that the oxidation of D-2-hydroxyacids by an enzyme present in rat tissues did not require nicotinamide adenine dinucleotide. Hellerman, Ross, Parmar, Wein & Lasser (1960) reported that D-malic acid was slowly oxidized by a preparation of succinic dehydrogenase obtained from pig heart in the absence of coenzyme. Tubbs & Greville (1961) found D-2-hydroxyacid-oxidase activity [D-2-hydroxyacid: cytochrome C oxidoreductase: E.C.1.1.2.4] in the mitochondria prepared from the liver and kidney of several animal species. Oxidation of a number of D-2-hydroxyacids, including D-malic acid, occurred in the presence of 2,6-dichlorophenolindophenol, ferricyanide and cytochrome C but not oxygen. Nicotinamide adenine dinucleotide was not required. The enzyme obtained from the mitochondria of rabbit-kidney cortex was partially purified. Britten (1968) has shown that oxalacetate, an intermediate of the citric-acid cycle, is formed when D-malic acid is incubated with the enzyme in the presence of ferricyanide. It is of additional interest

that Gamble (1965) found that rabbit-liver mitochondria accumulated both L- and D-malic acids against a concentration gradient.

In order to obtain additional experimental evidence to support the use of DL-malic acid as a food additive, the excretion of radioactivity has been measured in the urine, faeces and expired air of rats given [U-^{14}C]L- and [4-^{14}C]DL-malic acids by both oral and intraperitoneal administration.

EXPERIMENTAL

Materials. L- and DL-Malic acids and DL-aspartic acid were purchased from Koch-Light Laboratories, Colnbrook, Bucks. [U-^{14}C]L-malic acid (22·6 mC/m-mole) was obtained from the Radiochemical Centre, Amersham, Bucks. It was diluted with L-malic acid to give a specific activity of 61 μC/m-mole. [4-^{14}C]DL-aspartic acid (1·62 mC/m-mole) was obtained from the New England Nuclear Corporation, Boston, Mass. It was diluted with DL-aspartic acid to a specific activity of 65 μC/m-mole.

Preparation of [4-^{14}C]DL-malic acid. To a suspension of [4-^{14}C]DL-aspartic acid (342 mg) in water (8 ml) was added a solution of sodium nitrite (298 mg) in water (3 ml). The mixture was stirred continuously for 1 hr and a solution of lead acetate (950 mg) in water (2 ml) was added. The mixture was kept at 4°C for 18 hr and the precipitate of lead malate was collected. It was suspended in water (12 ml) and stirred for 20 min with the cation-exchange resin, Amberlite IR-120 (H^+-form). The mixture was then filtered and the filtrate was freeze-dried. The residue was recrystallized from an ethyl acetate–hexane mixture to give material (218 mg) with a specific activity of 93 μC/m-mole (expressed as malic acid).

A portion of this material was subjected to descending chromatography on Whatman chromatography paper (no. 1) using butan-1-ol/acetic acid/water (2:1:1·4, by vol.). When dry, the chromatogram was sprayed with an aqueous solution of bromophenol blue (0·04%). Only one component was detected and its R_F value (0·63) was identical with that given by authentic DL-malic acid.

The conversion of unlabelled DL-aspartic acid gave an optically inactive product.

Animals and dosing. Male albino Wistar rats (approximate body weight 200 g) of the Alderley Park SPF strain were given 0·5 ml of an aqueous solution of the appropriate acid, either by stomach tube or by intraperitoneal injection, the dose given being equivalent to 2·5 mg/kg. In the first group of six experiments on the two acids, data were recorded for 48 hr after administration, but subsequent tests were terminated after 24 hr.

Collection and assay of excreta. Animals were transferred to individual metabolism cages and urine was collected free from faeces. The cage was vented by a current of carbon dioxide-free air (500 ml/min) which then passed through a vertical glass column (30 × 300 mm) loosely packed with stainless-steel rings through which N-sodium hydroxide percolated from a reservoir at 30 ml/hr (Gage, 1963). Samples of the solution that emerged from the bottom of the column were taken at intervals over 24 or 48 hr and assayed for radioactivity by the method previously described (Daniel & Gage, 1965). Urine and faeces were collected at 24 hr and, in the early experiments, also at 48 hr.

RESULTS AND DISCUSSION

L- and DL-Malic acids were rapidly and extensively metabolized when administered to rats by either the oral or the intraperitoneal route (Table 1). In each case most of the radioactivity (83–92%) was excreted within 24 hr as carbon dioxide in the expired air. Table 2

shows the high proportion excreted by this route within 6 hr of oral administration. In the six early experiments it was found that only an additional 2% of the radioactivity was similarly excreted during the second day, and later experiments were therefore terminated after 24 hr. Only small amounts of radioactivity were found in the urine (3–9%) and faeces (0·3–1·4%).

Table 1. *The 24-hr excretion of radioactivity by rats given either* DL- *or* L-*malic acid orally or by intraperitoneal injection*

Malic acid	Route	Radioactivity (%) excreted in			
		Expired air	Urine	Faeces	Total
DL-	Oral	91·6 (88·1–94·0)	3·1 (2·4–3·8)	0·6 (0·0–0·9)	95·3
	Intraperitoneal	83·4 (80·6–85·3)	8·8 (8·5–9·0)	0·3 (0·1–0·6)	92·5
L-	Oral	88·0 (84·8–89·9)	3·2 (2·8–3·9)	1·4 (0·1–3·0)	92·6
	Intraperitoneal	86·6 (84·8–89·6)	3·1 (1·7–4·3)	1·4 (1·1–2·1)	91·1

Results are the average of three experiments, with figures in parentheses indicating the range of values obtained.

Table 2. *Mean hourly excretion rate of radioactivity in the expired air of rats dosed orally with either* DL- *or* L-*malic acid*

Malic acid	Excretion (%) at hr						Total
	1	2	3	4	5	6	
DL-	38·8	16·6	12·2	10·5	4·0	5·4	87·5
L-	45·3	16·1	11·0	4·6	4·0	3·4	84·4

As carbon dioxide is a gluconeogenetic substrate (Wood & Utter, 1965), some incorporation of radioactivity into products of normal intermediary metabolism is likely to occur. Microbiological breakdown in the gastro-intestinal tract appears to be of little or no importance, as is indicated by the fact that the route of administration had little effect upon the metabolism of L- or DL-malic acid. The somewhat greater urinary excretion of radioactivity from the DL form after intraperitoneal injection may be explicable on the basis of the finding (Vishwakarma & Lotspeich, 1960) that in chickens D-malic acid, unlike the L-isomer, is actively secreted by the renal tubules, although in these experiments only 36% of the acid was recovered from the urine.

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Le métabolisme des acides L- et DL-malique chez le rat

Résumé—Les acides L et DL-malique marqués au carbone 14 et administrés par voie orale ou intrapéritonéale à des rats ont tous deux été très largement métabolisés, la radioactivité étant excrétée à 90–95% dans les 24 heures, principalement sous la forme de gaz carbonique-^{14}C dans l'air expiré (83 à 92% de la radioactivité administrée). De plus, les acides ont été métabolisés à un même degré, indépendant de la voie d'administration. Il ne semble donc y avoir aucun motif de proscrire l'addition d'acide D-malique aux aliments.

Der Stoffwechsel von L- und DL-Maleinsäure in Ratten

Zusammenfassung—Bei oraler und intraperitonealer Verabreichung von mit ^{14}C markierter L- oder DL-Maleinsäure an Ratten wurden die Verbindungen im Stoffwechsel gründlich umgesetzt und 90–99% der Radioaktivität innerhalb von 24 Stunden ausgeschieden, und zwar hauptsächlich in der ausgeatmeten Luft als [^{14}C]-Kohlendioxyd (83–92% der verabreichten Radioaktivität). Ausserdem wurden die Säuren mit der gleichen Geschwindigkeit umgesetzt, die sich als unabhängig vom Verabreichungswege erwies. Es scheint somit kein Grund gegen die Verwendung von D-Maleinsäure als Lebensmittelzusatz zu sprechen.

(Italian pp. 1455-7)

D-aspartate oxidase from pig's kidney

II. Inhibition by malic acid and tartaric acid

By C. de Marco

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Cagliari Chapter - Session of June 27, 1966

Recently a method was developed for preparing from pig's kidney D-aspartate oxidase (D-aspartate: O_2 oxidoreductase, deaminant, E.C. 1.4.3.1) partially purified (1). This enzyme, evidenced by Still et al. (2, 3), differs from D-aminoacid oxidase (D-aminoacid: O_2 oxidoreductase, deaminant, E.C. 1.4.3.3), not only because of the substrate specificity, but also because unlike the latter it is not inhibited by benzoic acid.

We believed it to be interesting to search for compounds which could exert a competitive inhibition of aspartic acid, so that we could have some preliminary indications on the chemical and stereochemical properties that cause the affinity of the substrate to the enzyme.

The D-aspartate oxidase was prepared from pig kidney as previously described; we used enzyme in the 2nd or 4th state of fractionation (1). The activity was determined according to the consumption of oxygen in the Warburg, at 38°C, at pH 8.3, in the presence of FAD and catalysis.

As Dixon and Kleppe (4) evidenced that some L-aminoacids exert an inhibiting action on D-aminoacid oxidase, we studied in particular the action of L-aspartic acid on the oxidation of D-aspartic acid. It was demonstrated that the L isomer has no inhibiting action whatever, at equal or double concentration relative to the substrate.

Dixon and Kleppe also showed that several alpha-oxyacids exert an inhibiting action on D-aminoacid oxidase. We therefore wanted to test the action of the corresponding oxyacid, malic acid, on the oxidation of aspartic acid.

In fig. 1 is shown the time response of the oxidation of D-alanine (as substrate for D-aminoacid oxidase) and of D-aspartic acid, in the presence and absence of DL-malic acid. As enzymatic prepration there was used in that case a fraction still having D-aminoacid oxidase activity (stage 2, cf. 1). It is evident that malic acid has no effect on the rate of oxidation of the alanine; this is in agreement with the results of Dixon and Kleppe. This oxyacid is, in fact, the only one of those tested by them which has no inhibiting action on D-aminoacid oxidase.

Fig. 1. Effect of DL-malic acid on the oxidation of D-alanine and of D-aspartic acid.

O_2 consumption observed in the Warburg. In each tray: 20 mg enzyme (stage 2, cf. 1); 30 mgc FAD; 50 mcg catalase; 1 ml pyrophosphate of Na 0.1 M pH 8.3. Final volume 3 ml. Temp. 38°C. Gas: air.

Curve 1: Full dots: 30 micro-mol D-alanine; empty dots: 30 micro-mol D-alanine + 60 micro-mol DL-malic acid.

Curve 2: 30 micro-mol D-aspartic acid.

Curve 3: 30 micro-mol D-aspartic acid + 60 micro-mol DL-malic acid.

It is evident from the same figure, however, that the oxidation of the aspartic acid is considerably slowed by the presence of malic acid. Using enzyme in the 4th state of fractionation, that is, entirely devoid of D-aminoacid oxidase activity, some tests were then carried out to determine the type of inhibition caused by the malic acid. The results obtained, given in fig. 2 in a plot according to Lineweaver and Burk, clearly show that the malic acid exerts a competitive type inhibition. The Ki values were not calculated, as it was expected that the activities of the D and L-malic acids could be tested separately.

Fig. 2. Competitive nature of the inhibition of the D-aspartate oxidase by malic acid. The activity of the enzyme was observed in the Warburg.

In each tray: 7 mg enzyme (stage 4, cf. 1); 20 mcg FAD; 50 mcg catalase; 1 ml pyrophosphate of Na 0.1 M pH 8.3.

Final volume 3 ml. Temp. $38^{\circ}C$. Gas: air.

v = /ul O_2/min. S = D-aspartate 10^{-2} M.

DL-malic acid = 2.10^{-2} M.

Some preliminary tests with the D and L-tartaric acids showed that also the latter exerts an inhibiting action of the same order as that exerted by malic acid. The D-tartaric acid, however, is entirely inactive.

These preliminary results seem to us to be interesting both because the inhibition by malic acid constitutes a further differentiation between D-aminoacid oxidase and D-aspartate oxidase, and because the study of the inhibition by malic and tartaric acid can furnish indications on the configurations causing the stereo-specificity of D-aspartate oxidase.

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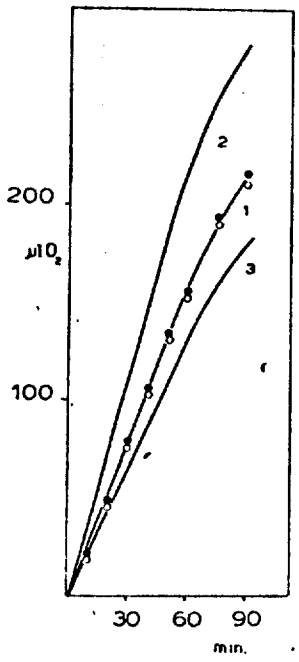
200
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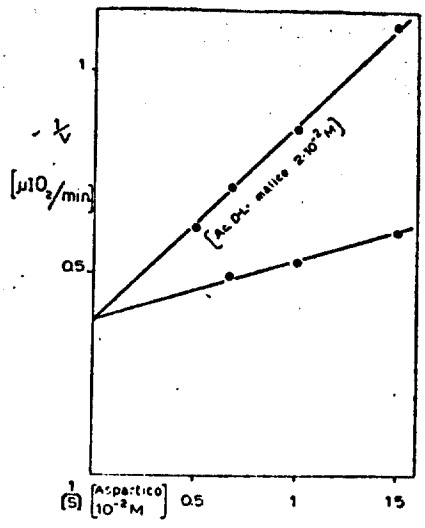
Fig. 1


1
0.5
- 1/v
[µl0₂/min]
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1/(S) [Aspartico 10⁻² M]
0.5
1
1.5

Fig. 2

TABELLA. — *Aumento dell'attività nor-adrenalino liberatrice della tiramina somministrata per os ad opera della pargilina, nel ratto.*

Trattamento (*) mg/kg di peso			Nor-adrenalina cardiaca (μg/g di tessuto) 4 h dopo la somministrazione dell'agente depletizzante (***)
Controlli	—	—	0,83 — (0,80 - 0,85)
Tiramina HCl	—	12,5	0,77 — (0,75 - 0,78)
»	—	25,0	0,65 — (0,61 - 0,67)
»	—	50,0	0,48 — (0,46 - 0,50)
Pargilina	80,0 +	—	0,94 — (0,93 - 0,95)
Pargilina - tiramina HCl	80,0 +	12,5	0,92 — (0,86 - 0,95)
» + »	80,0 +	25,0	0,56 — (0,40 - 0,52)
» + »	80,0 +	50,0	0,21 — (0,18 - 0,25)
Pargilina - DMI - tiramina HCl (**)	80,0 +	10,0 + 25,0	0,80 — (0,75 - 0,87)

(*) La tiramina diluita in soluzione fisiologica è stata somministrata per *os*. 1 ml/100 g di peso corporeo. La pargilina venne somministrata in peritoneo 24 h prima della tiramina.

(**) Il DMI è stato somministrato i.p. 20 min prima della tiramina.

(***) Ogni valore è la media di 5 determinazioni. In parentesi valori estremi.

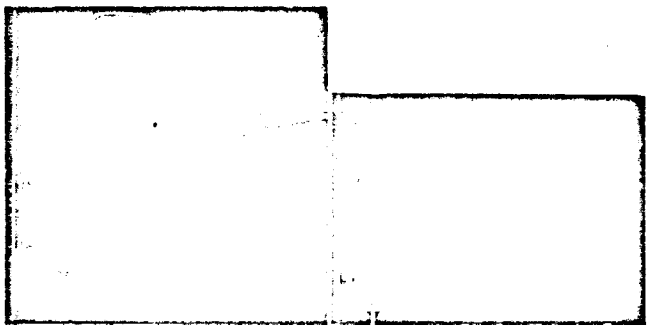

Fig. 2.

A B

A) Ratto pretrattato con pargilina alla dose di 80 mg/kg i.p., 1 h prima della tiramina. T. = tiramina. HCl 500 μg i.v.
DMI = desmetilimipramina 1 mg/kg i.v.

B) Ratto pretrattato con pargilina alla dose di 80 mg/kg e DMI alla dose di 1 mg/kg i.v., rispettivamente 1 h prima e 20 min prima della somministrazione della tiramina. T. = tiramina. HCl 500 μg i.v.

Infine, come si vede dalla fig. 2, il DMI non solo è capace di impedire l'effetto ipertensivo della tiramina, ma è capace anche di antagonizzare tale effetto quando esso sia già in atto.

Questi nostri risultati indicano come nella pratica clinica un'associazione di entrambi gli antidepressivi pargilina e DMI possa evitare i pericoli di un'accidentale ingestione di tiramina.

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D-ASPARTATO OSSIDASI DAL RENE DI MAIALE. — II) INIBIZIONE DA ACIDO MALICO E TARTARICO. — C. De Marco.

(*Dagli Istituti di Fisiologia dell'Università di Cagliari e di Chimica Biologica dell'Università di Roma*).

Sezione di Cagliari — Seduta del 27 giugno 1966.

Recentemente è stata messa a punto una metodica per preparare dal rene di maiale la D-aspartato ossidasi (D-aspartato: O_2 ossidoreduttasi, deaminante. E.C. 1.4.3.1) parzialmente purificata (1). Questo enzima messo in evidenza da Still e coll. (2, 3), si differenzia dalla D-aminoacido ossidasi (D-aminoacido: O_2 ossidoreduttasi. deaminante. E.C. 1.4.3.3). oltrechè per la specificità di substrato. perchè a differenza di quest'ultimo non viene inibito dall'acido benzoico.

Ci è parso interessante ricercare eventuali composti che potessero esplicare una inibizione competitiva verso l'acido aspartico. per avere alcune indicazioni preliminari sulle proprietà chimiche e stereochimiche che condizionano la affinità del substrato verso l'enzima.

La D-aspartato ossidasi è stata preparata dal rene di maiale come precedentemente descrito; si è usato enzima al 2° o al 1° stadio di frazionamento (1). L'attività è stata determinata seguendo il consumo di ossigeno al Warburg. a 38°C, a pH 8,3 in presenza di FAD e catalasi.

Poichè Dixon e Kleppe (4) hanno messo in evidenza che alcuni L-aminoacidi esplicano azione inibente sulla D-aminoacido ossidasi, abbiamo innanzitutto studiato l'azione dell'acido L-aspartico sulla ossidazione del D-aspartico. Si è dimostrato che l'isomero L non ha alcuna azione inibente, a concentrazione eguale o doppia rispetto al substrato.

Gli stessi Dixon e Kleppe hanno dimostrato inoltre che diversi α-ossiacidi esplicano anzione inibente sulla D-aminoacido ossidasi. Abbiamo pertanto voluto saggiare l'azione sulla ossidazione dell'acido aspartico, dell'ossiacido corrispondente, l'acido malico. Nella fig. 1 è riportato l'andamento nel tempo della ossidazione della D-alanina (come substrato per D-aminoacido ossidasi) e dell'acido D-aspartico, in presenza ed in assenza di acido DL-malico. Come preparato enzimatico è stata usata in tal caso una frazione

avente ancora attività D–aminoacido ossidasica (stadio 2°, cfr. 1). E' evidente che l'acido malico non ha alcuna influenza sulla velocità di ossidazione della alanina: ciò è in accordo con i risultati di Dixon e Kleppe. Tale ossiacido infatti è l'unico tra quelli da essi provati, che non ha alcuni azione inibente sulla D–aminoacido ossidasi.

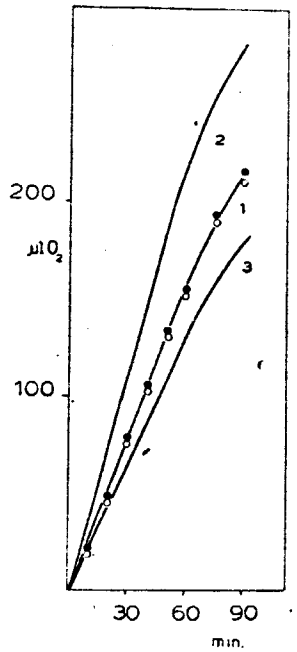

Fig. 1. — *Effetto dell'acido DL-malico sulla ossidazione della D-alanina e dell'acido D-aspartico.*
Consumo di O_2 seguito al Warburg. In ogni vaschetta: 20 mg enzima (stadio 2°, cfr. 1); 30 µg FAD; 50 µg catalasi; 1 ml pirofosfato di Na 0,1 M pH 8,3.
Volume finale 3 ml. Temp. 38°C. Gas: aria.
Curva 1: tondi pieni: 30 µ-mol D-alanina,
tondi vuoti: 30 µ-mol D-alanina + 60 µ-mol acido DL-malico.
Curva 2: 30 µ-mol acido D-aspartico.
Curva 3: 30 µ-mol acido D-aspartico + 60 µ-mol acido DL-malico.

Dalla stessa figura risulta invece evidente che l'ossidazione dell'acido aspartico è notevolmente rallentata dalla presenza di acido malico. Utilizzando enzima al 4° stadio di frazionamento, cioè del tutto privo di attività D–aminoacido assidasica, si sono quindi effettuate alcune prove volte a determinare il tipo di inibizione causata dall'acido malico. I risultati ottenuti, riportati nella fig. 2 in un «plot» secondo Lineweaver e Burk, dimostrano chiaramente

che l'acido malico esplica una inibizione di tipo competitivo. Non sono stati calcolati i valori di Ki, in attesa di poter saggiare separatamente l'attività degli acidi D ed L–malico.

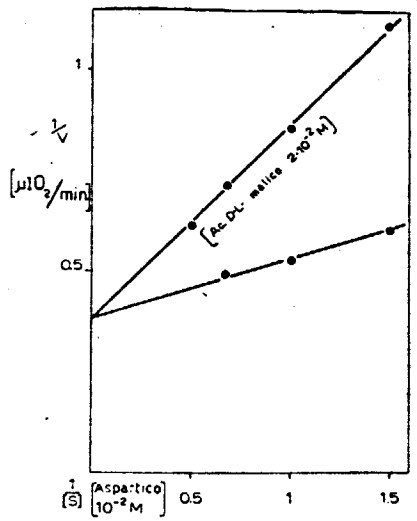

Fig. 2. — *Natura competiva della inibizione della D-aspartato ossidasi da acido malico.*
L'attività dell'enzima è stata seguita al Warburg.
In ogni vaschetta: 7 mg enzima (stadio 4°, cfr. 1); 20 µg FAD; 50 µg catalasi; 1 ml pirofosfato di Na 0,1 M pH 8,3.
Volume finale 3 ml. Temp. 38°C. Gas: aria.
v = µl O_2/min. S = D-aspartato 10^{-2} M.
Acido DL-malico = 2.10^{-2} M.

Alcune prove preliminari con gli acidi D ed L–tartarico hanno dimostrato che anche quest'ultimo esplica una azione inibente dello stesso ordine di quella esplicata dall'acido malico. L'acido D–tartarico è invece del tutto inattivo.

Questi risultati preliminari ci paiono interessanti sia perchè l'inibizione da acido malico costituisce una ulteriore differenziazione tra D–aminoacido ossidasi e D–aspartato osidasi, sia perchè lo studio della inibizione da acido malico e tartarico potrà fornire indicazioni sulle configurazioni condizionanti la stereospecificità della D–aspartato ossidasi.

(1) De Marco C. e coll., G. Biochim., in stampa.
(2) Still J. L. e coll., J. Biol. Chem., 1949, 179, 831.
(3) Still J. L., Sperling E., J. Biol. Chem., 1950, 182, 585.
(4) Dixon M., Kleppe K., Biochim. Biophys. Acta, 1965, 96, 368.

Formation of α-ketoglutaric acid in the animal metabolism

By P. E. Simola and F. E. Krusius

(Received on Feb. 15, 1938)

In previous investigations, one of us (Simola (1)) had studies the role of α-glutaric acid in the animal metabolism. It was found for the first time in 1936 that large amounts of α-ketoglutaric acid are excreted in the rat urine of certain B-vitamin components are missing. This indicate in turn that certain B-vitamin components, particularly B1, must be related in a certain sense to the keto acid metabolism in the entire organism, and not only in the brain. On the other hand, it was found in 1937 that normal rats excrete large amounts of α-ketoglutaric acid in the urine after peroral administration of pyroracemic acid. These load tests proved exactly, probably for the first time, the importance of α-ketoglutaric acid as an intermediate product in the carbohydrate metabolism.

In order to obtain more information about the formation and reaction of α-ketoglutaric acid in the animal organism, it seemed necessary to study the excretion of keto acids after peroral administration of some biologically important substances, like lactic-, oxalacetic- and citric acid. To this end one of us (Krusius) has tried for a long time to develop quantitative methods of determination of α-keto acids, particularly of pyroracemic and α-ketoglutaric acid, which will be reported elsewhere.

In our investigations of the excretion of α-keto acids which were carried out as before with rats as test anaimals, we found only minor changes in the excretion of pyroracemic acid after peroral administration of lactic-, succinic-, fumaric-malic-, citric- and α-ketoglutaric acid. With regard to the excretion of α-ketoglutaric acid, we found that considerable amounts were excreted again under load with the acid itself. Strangely enough large amounts of α-ketoglutaric acid are also excreted under load with succinic-, fumaric-, malic- and citric acid. After

administration of lactic acid, no marked changes of the normal α-keto-glutaric acid were observed in the urine. Our tests on the loading with oxalacetic acid are not yet completed.

Apart from the experimental additional proof of the importance of α-ketoglutaric acid, these tests show that the vegetable acids succinic, fumaric-, and malic acid serve as effective intermediate products in the metabolism and can actually be reacted further in large amounts, and that the citric acid reaction is very closely related to the carbohydrate metabolism.

We can not go into details here about the mechanism of formation and certain hypotheses that appeared in the literature on the reaction of α-ketoglutaric acid. The presence of other metabolites in the urine after administration of the above mentioned acids will be reported later.

Medical-Chemical Laboratory of the University of Helsinki.
Helsinki, Feb. 12, 1938.

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(1) P. E. Simola: Suomen Kemistilehti B, IX, 19-20, 1936; B, X, 19, 1937. Paper delivered at the 19th Scandinavian Scientists Convention in Helsinki 1936 and at the 5th Nordic Convention of Physiology in Upsala 1937.

(Translated by Carl Demrick Associates, Inc./IE)

d^{20}_4 ... daraus $d^{20.2}_4$ = 1.0118. ... n^{20}_D = 1.4624.
M_D ... für $C_{11}H_{18}O_2^{"}O_2 <F$ 55.88, gef. 56.55. EM = + 0.67.
EΣ ... 31

...tiv-kinetische Untersuchungen über die ...ationsgeschwindigkeiten der drei Tanaceton- ...säuredimethylester sind schon ausgeführt wo... nu werden, wie auch die Untersuchung der Kon...ationsprodukte fortgesetzt.

Ch...isches Laboratorium der Universität Helsinki.

1) Ann. Acad. Scient. Fenn. A, XXVII; N:o 8 (1928).
2) ... 30. 155 (1897), vgl. auch 31. 2512 (1898).
3) ...tation (Göttingen 1909); S. 76–78. Journ. Chem. So... ...08 (1910).
4) ... 23, Fussnote.

D...utung von α-Ketoglutarsäure im tierischen Stoffwechsel.

P. E. Sim... und *E.-E. Kr...s*

(...gegangen am 15. Februar 1938.)

... Untersuchungen hat schon der eine von ...nal¹) die ... der α-Ketoglutarsäure im ... Stoffwechsel behandelt. Es wurde zuerst ... festgestellt, dass bei Rattenharn beim Ausschluss gewisser B-Vitaminkomponenten grosse Mengen α-Ketoglutarsäure ausgeschieden werden. Dies wies ... darauf hin, dass bestimmte B-Vitaminkomponenten, vor allem B_1, in gewisser Beziehung zum ...säurenstoffwechsel im ganzen Organismus und nicht nur im Gehirn stehen müssen. Andererseits wurde 1937 auch festgestellt, dass normale Ratten nach peroraler Brenztraubensäureverabreichung grosse Mengen α-Ketoglutarsäure im Harn ausscheiden. Durch diese Belastungsversuche wurde die Bedeutung der α-Ketoglutarsäure als normales Durchgangsglied im Kohlenhydratstoffwechsel wohl zum ersten Male exakt experimentell bewiesen.

Um näheren Aufschluss zu erhalten über die Entstehung und Umsetzung der α-Ketoglutarsäure im Tierorganismus, erschien es ... nötig die Ausscheidung von Ketosäuren nach ... der Verabreichung einiger biologisch wichtiger Stoffe, wie Milch-, Bernstein-, Fumar-, Äpfel-, Oxalessig- und Citronensäure zu untersuchen. Zu diesem Zwecke hat der eine von uns (*Krusius*) sich schon längerer Zeit bemüht die quantitativen Bestimmungsmethoden der α-Ketosäuren, vor allem der Brenztrauben- und α-Ketoglutarsäure zu entwickeln, worüber getrennt berichtet wird.

In unseren Untersuchungen über die α-Ketosäurenausscheidung, die wie auch früher mit Ratten als Versuchstieren ausgeführt wurden, fanden wir, dass nach peroraler Verabreichung der Milch-, Bernstein-, Fumar-, Äpfel-, Citronen- und α-Ketoglutarsäure nur geringfügige Veränderungen der Brenztraubensäureausscheidung festzustellen waren. Betreffend die α-Ketoglutarsäureausscheidung haben wir gefunden, dass bei Belastung mit der Säure selbst bedeutende Mengen wieder ausgeschieden werden. Merkwürdigerweise werden aber auch nach Belastung mit Bernstein-, Fumar-, Äpfel- und Citronensäure grosse Mengen α-Ketoglutarsäure ausgeschieden. Nach Verabreichung mit Milchsäure waren keine deutlichen Veränderungen des normalen α-Ketoglutarsäuregehaltes im Harn zu beobachten. Unsere Untersuchungen über Belastung mit Oxalessigsäure sind nicht beendigt.

Ausser dem experimentellen weiteren Beweis der Bedeutung der α-Ketoglutarsäure im intermediären Stoffwechsel zeigen diese Versuche, dass die Pflanzensäuren Bernstein-, Fumar-, und Äpfelsäure als wirkliche Zwischenprodukte im Stoffwechsel dienen und tatsächlich in grossen Mengen weiter umgesetzt werden können, und dass der Citronensäureumsatz mit dem Kohlenhydratstoffwechsel sehr nahe verknüpft ist.

Auf den Entstehungsmechanismus und gewisse in der Literatur erschienene Hypothesen über den α-Ketoglutarsäureumsatz können wir in diesem Zusammenhang nicht eingehen. Auch über das Vorkommen anderer Stoffwechselprodukte im Harn nach Verabreichung der obengenannten Säuren wird, was später berichtet werden.

Medizinisch-chemisches Laboratorium der Universität Helsinki.

Helsinki den 12. Februar 1938.

1) *P. E. ...nal* Suomen Kemistilehti B, IX, 19...30,1936; B X, 19, ... Vortrag auf dem 19. Skandinavischen Naturforscherkongress in Helsinki 1936 und auf dem 5. Nordischen Kongress für Physiologie in Upsala 1937.

Über die Kondensation des δ-Phenyllävulinsäuremethylesters mit Natriummethylat.

(Vorläufige Mitteilung).

Salli Es...

(Eingegangen am 15. Februar 1938.)

... ... substituierte ...säureester, ... gegen Behauptungen von *Le...* und *...r Rosan...* ziemlich leicht mit Natrium... unter ... eines Diketocyclopentan... kondensieren lassen. Um die begonnene Arbeit zu vervollständigen und den Einfluss der Phenylgruppe in δ-Stellung zur Carbomethoxylgruppe auf die Kondensation zu ..., wurde diese mit δ-Phenyllävulinsäuremethylester ausgeführt. Der fragliche Ester wurde nach der Methode von *Gertrude Maud Ro...* dargestellt.

...642 ... Substanz: 14.50... ... 5.430 mg H_2O
...832 ... : 9.760 ... 2.275 „ „

	Gef.		Ber. f. $C_{12}H_{14}O_3$
	I	II	
% C	70....	70.16	...50
% H	6.8...	6.94	...85

Der Ester wurde zuerst in methylalkoholischer Lösung mit Natriummethylat (1.5 Mole $NaOCH_3$ für 1 Mol Ester) auf dem Wasserbade erwärmt und nach Abdestillieren des Methanols noch einige ... bei höherer Temperatur 150-160° gehalten. Nach dem ... der wässrigen alkalischen Lösung wurde eine weisse ... gewonnen, die, aus Eisessig umkrystallisiert, sich ... bräunte und bei 233—234° schmolz. Die Ausbeute an Rohprodukt war sehr klein ca. 15 % der Theorie.

6,045 mg Substanz: 16,765 mg CO_2 und 3,050 mg H_2O

	Gef.	Ber. f. $C_{11}H_{10}O_2$
% C	75,62	75,83
% H	5,65	5,79

STUDIES ON THE CARBOHYDRATE METABOLISM OF SHEEP

VIII. THE INTERPRETATION OF CHANGES IN THE LEVELS OF METABOLIC INTERMEDIATES AFTER INTRAVENOUS INJECTION OF PROPIONATE, SUCCINATE, AND MALATE

By R. L. Reid* and S. C. Mills*

[*Manuscript received December* 12, 1960]

Summary

Blood levels of pyruvic, citric, and α-oxoglutaric acids in sheep are similar to those in man. Citric acid declines markedly on fasting; levels in ewes with pregnancy toxaemia are not significantly different from those in fasted, pregnant ewes. The presence of low citric acid levels in ewes with pregnancy toxaemia in which blood glucose levels are normal or above may further support the suggestion that there is an interference with glucose utilization in pregnancy toxaemia.

Intravenous injection of propionate, succinate, and malate into fasted, pregnant ewes always lowered blood ketones, but it is not known to what extent this response is merely a consequence of increased blood glucose following injection. Injected propionate disappeared more rapidly than injected acetate.

Propionate injection into fed, non-pregnant ewes is followed by an immediate increase in blood glucose to high levels; the disappearance of excess glucose appears to follow the pattern of a normal glucose tolerance curve. Blood pyruvic acid increases markedly with blood glucose. Succinate and malate injections are followed by small increases in blood glucose and pyruvic acid. The difficulty of interpreting changes in blood glucose in inadequately trained experimental animals is emphasized.

Consistent, significant increases in blood oxalacetic acid did not occur in response to either propionate, succinate, or malate injection. Blood citric and α-oxoglutaric acids increased markedly after succinate and malate, but not after propionate injection.

The data are discussed in relation to metabolic pathways in sheep tissues; it is concluded that this type of *in vivo* experiment is of limited value.

I. Introduction

The studies reported here began in investigations several years ago into the possible role of oxalacetate insufficiency as a cause of moderate ketosis in undernourished pregnant ewes (Reid and Hogan 1959). The observation that blood citric acid levels increased under conditions probably associated with a stimulation of tricarboxylic acid cycle activity, following either feeding (Reid and Hogan 1959) or glycerol administration to hyperketonaemic ewes (Reid 1960*a*), led to investigation of the effects of intravenous injection of the oxalacetate precursors succinate and malate, and also of propionate, on hyperketonaemia and on blood citrate levels. Anomalous results with propionate led to closer investigation of changes in blood levels of several metabolic intermediates following injection of propionate, malate, or succinate. The results of these studies are presented in this paper.

* Division of Animal Physiology, C.S.I.R.O., Ian Clunies Ross Animal Research Laboratory, Prospect, N.S.W.

II. Experimental Methods

Experiments were carried out on Merino or Corriedale ewes. Injection experiments on fed sheep were carried out in the morning, before feeding. Propionate, succinate, and malate (free acids in 1M aqueous solution neutralized to pH 7·4 with sodium hydroxide) were injected into an external jugular vein at dose rates of 2 or 2·25 m-moles per kg body weight, over a period of 2 min. Post-injection blood samples were obtained from the external jugular vein on the opposite side.

All analyses were on whole blood. Analyses for glucose, ketone bodies, volatile fatty acids (V.F.A.), and citric acid were carried out on blood collected with potassium oxalate as anticoagulant; samples were analysed immediately, or after storage at −20°C. Glucose was determined by the method of Somogyi (1937, 1952), ketone bodies by the method of Green and Eden (Reid 1960*b*), and V.F.A. by a modification of McClendon's (1944) microdistillation procedure (Reid 1950*b*). Citric acid was estimated by a modification of the method of Speck, Moulder, and Evans (1946) as outlined by Reid and Hogan (1959). The errors of these methods have been discussed elsewhere (Reid 1950*a*, 1960*b*; Reid and Hogan 1959).

Pyruvic acid and total keto-acids were determined by the method of Friedemann and Haugen (1943) on blood measured directly into cold 10% (w/v) trichloroacetic acid. At the same molar concentrations as pyruvate, the errors introduced into the pyruvate method (xylene extraction; readings at 320 mμ) by the presence of α-oxoglutarate, acetoacetate, and oxalacetate were 12, 16, and 25% respectively. But, at the highest concentrations of these three keto-acids actually encountered, the maximum errors caused by each in the estimation of pyruvate were respectively +0·25, +0·1, and +0·1 mg pyruvic acid per 100 ml blood (mg %). In experiments where α-oxoglutaric acid was determined, pyruvic acid levels have been corrected for α-oxoglutarate interference. Acetoacetate caused negligible interference in the estimation of total keto-acids (ethyl acetate extraction).

Blood for α-oxoglutaric acid estimation was collected directly into metaphosphoric acid (10% w/v) solution. Keto-acid hydrazones were extracted by the method of el Hawary and Thompson (1953) and separated chromatographically on paper dipped in 0·15M phosphate buffer at pH 6·2 (Isherwood and Cruickshank 1954). Recovery of α-oxoglutarate added to blood was 80%.

Oxalacetic acid was determined on blood collected directly into an equal volume of 6% (w/v) trichloroacetic acid cooled to just above freezing point in a dry ice-alcohol mixture. The method was that of Kalnitsky and Tepley (1958); recovery of oxalacetate added to blood was 80%. Previous attempts to estimate oxalacetic acid after chromatographic separation of the keto-acid hydrazones were unsatisfactory, because of the variable amount of decarboxylation to pyruvic acid in the presence of amounts of pyruvic acid which were themselves varying during the experiment.

III. Results

(*a*) *Blood Levels of Pyruvic, Citric, Oxalacetic, and α-Oxoglutaric Acids in Sheep*

Mean prefeeding levels of glucose, ketones, and V.F.A. in the blood of 24 non-pregnant ewes fed on various diets were previously given as 43±1·2, 1·5±0·2, and

1·2 - 0·2 mg % (ketones as acetone) respectively (Reid 1960*c*). Most of these analyses were on ewes fed on roughage diets consisting of various proportions of chaffed wheaten and lucerne hays. Mean levels of pyruvic, citric, oxalacetic, and α-oxoglutaric acids in blood collected prior to feeding from the same or similar ewes fed on similar intakes of similar diets were 1·0±0·5, 3·0±0·25, 0·16±0·02, and 0·38±0·12 mg % respectively (Table 1).

Citric acid levels declined during fasting. Too few figures are available from non-pregnant ewes for inclusion in Table 1, but levels of 0·8–2·2 mg % have been recorded. It is not known whether levels are higher during fasting in non-pregnant

TABLE 1

BLOOD LEVELS OF PYRUVIC, CITRIC, OXALACETIC, AND α-OXOGLUTARIC ACIDS IN SHEEP

Analysis	No. of Analyses	No. of Ewes	Range (mg %)	Mean±S.E. (mg %)	Mean Blood Glucose±S.E. (mg %)
Pyruvic acid:					
Fed, non-pregnant ewes*	50	28	0·5 –2·0	1·0 ±0·05	40·0±0·9
Citric acid:					
Fed, non-pregnant ewes*	23	16	1·4 –6·8	3·0 ±0·25	40·6±1·3
Fed, pregnant ewes*	17	17	1·2 –6·5	3·4 ±0·32	39·9±1·4
Fasted, pregnant ewes*	20	15	0·4 –1·4	0·9 ±0·07	22·7±1·4
Ewes with pregnancy toxaemia:					
Induced in laboratory*	35	12	0·6 –2·1	1·0 ±0·08	20·4±2·4
Field cases	28	14	0·5 –1·8	0·9 ±0·08	35·3±6·1
Oxalacetic acid:					
Fed, non-pregnant ewes†	17	9	0·05–0·25	0·16±0·02	—
α-Oxoglutaric acid:					
Fed, non-pregnant ewes†	8	4	0·10–0·60	0·38±0·12	—

* Most analyses on ewes fed on roughage diets consisting of various proportions of chaffed wheaten and lucerne hays.

† All analyses on ewes fed on equal parts chaffed wheaten and lucerne hays.

than in pregnant ewes; too few comparisons have been made under the same experimental conditions. It should be noted that values in ewes with pregnancy toxaemia are not different from those in fasted, pregnant ewes.

(*b*) *Propionate, Succinate, and Malate Injections into Fasted, Pregnant Ewes*

The effects of injected propionate and succinate on blood glucose, ketone, and citrate levels in ewes fasted in late pregnancy were studied in "reversal" experiments on two ewes on the same two days (Figs. 1, 2).

Injected propionate disappeared from the blood within 45 min (Fig. 1). Unlike that of acetate (Reid 1958), its disappearance did not appear to follow an exponential function with respect to time. The concentration of blood glucose increased very rapidly after propionate injection to levels slightly above normal; blood ketone concentration declined appreciably (Fig. 2). Blood glucose increased more slowly

Experiment 3

Increases in blood levels of keto-acids other than pyruvic acid, following succinate and malate injections, could obviously be due to increases in blood oxalacetic acid. Alternatively, in view of the marked increases in citric acid levels, they could be due to increases in α-oxoglutaric acid.

Experiment 3 was carried out to investigate these possibilities; the observations made are presented in Figure 8. It was originally intended to compare the effects of saline, propionate, and malate injections in the same two ewes. This proved impracticable, as ewe 76 died following the injection of supposed *l*-malate from a new batch which subsequently proved to be not as labelled. Ewe 99, from the same group of

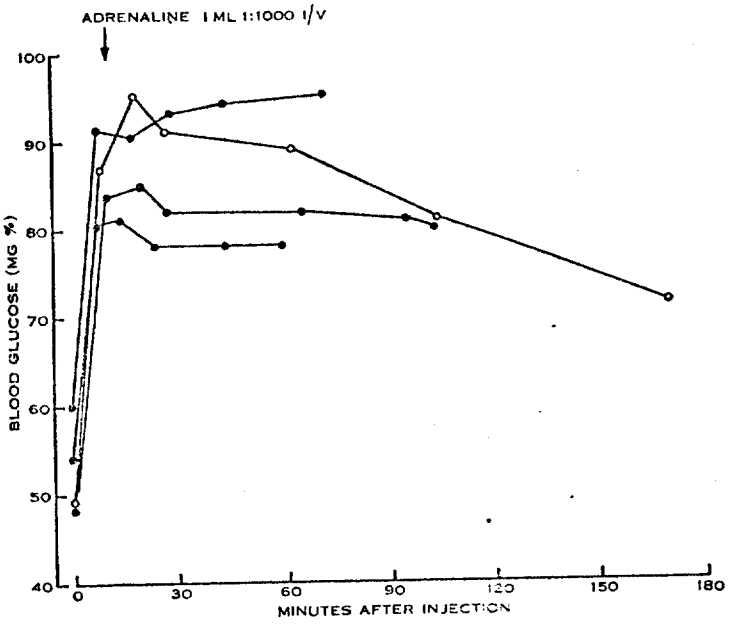


Fig. 7.—Blood glucose following intravenous injection of adrenaline in four experiments on two trained non-pregnant ewes.

ewes, and on the same ration (20 g per kg body weight of equal parts chaffed wheaten and lucerne hays) was included to complete the experiments. A previous succinate injection experiment on ewe 76 is included for comparison.

Saline injections were followed by slight, though definite increases in blood glucose level, but no significant changes in other blood constituents studied (Fig. 8).

As in experiment 1, blood glucose increased to high levels after propionate, returned to pre-injection levels in 1 hr, and then declined below pre-injection levels. Smaller increases in glucose level following malate and succinate tended to be maintained during the 2 hr of the experiments.

Pyruvic acid increases after propionate injection were not as great as in experiment 1, but were greater than those following malate injection.

As anticipated, changes following malate and succinate injections were very similar. Increases in levels of blood keto-acids other than pyruvic, and in citric acid, were marked, in contrast to small increases following propionate injection. Blood glucose and pyruvate increased rapidly to high levels after propionate injection.

In contrast to experiment 1 (Fig. 4), blood glucose did not return to pre-injection levels following propionate injection. These ewes were accustomed to handling and to other experimental procedures, but not to the procedures associated with injection experiments; saline injections in two ewes were followed by substantial increases in blood glucose (Fig. 6).

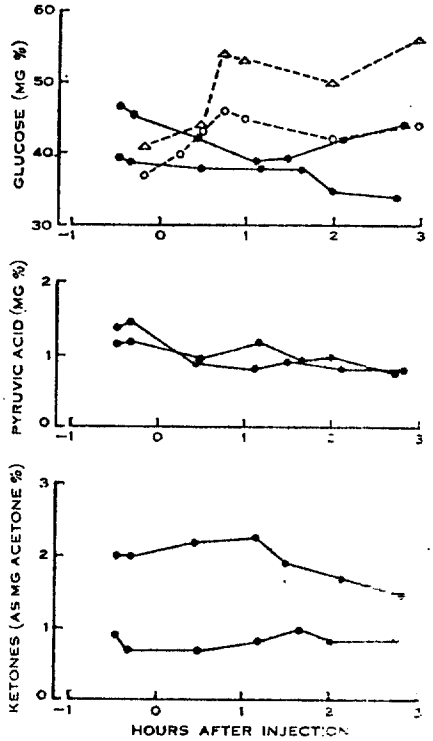


Fig. 6.—Effect of intravenous saline injection on blood glucose, pyruvic acid, and ketones. ●———● Ewes from same group as those in experiment 1 (Fig. 4); ○- - - -○ ewe which received malate in experiment 2 (Fig. 5); △- - - -△ ewe which received succinate in experiment 2 (Fig. 5).

In view of these observations, the effect of injected adrenaline on blood glucose is of interest (Fig. 7). In four experiments on two trained ewes, blood glucose increased from pre-injection levels of 48–57 mg % to 80–90 mg % within 8–11 min; these high levels were then maintained for at least 60–170 min. These results are similar to those obtained by Schultze (1959) on calves.

The substantial sustained increase in blood glucose after saline injection (Fig. 6) may obviously be a result of stimulation of the sympatho-adrenal system in these ewes; blood glucose changes in the same or similar ewes after propionate, succinate, and malate injections (Fig. 5) are thus difficult to interpret.

Very rapid removal of propionate was accompanied by a rapid increase in blood glucose, to levels much higher than recorded in fasted pregnant ewes. The removal of excess glucose after 30 min appeared to follow the pattern of a normal glucose tolerance curve, glucose levels declining to below pre-injection levels at 2 hr, as in glucose tolerance tests in which glucose removal was also rapid (Reid 1958). Increases in blood glucose level following succinate injection were less than in fasted pregnant ewes; glucose declined after reaching a peak only 15 min after injection. In contrast to those obtained with fasted pregnant ewes, increases in glucose after both propionate and succinate injection were not sustained.

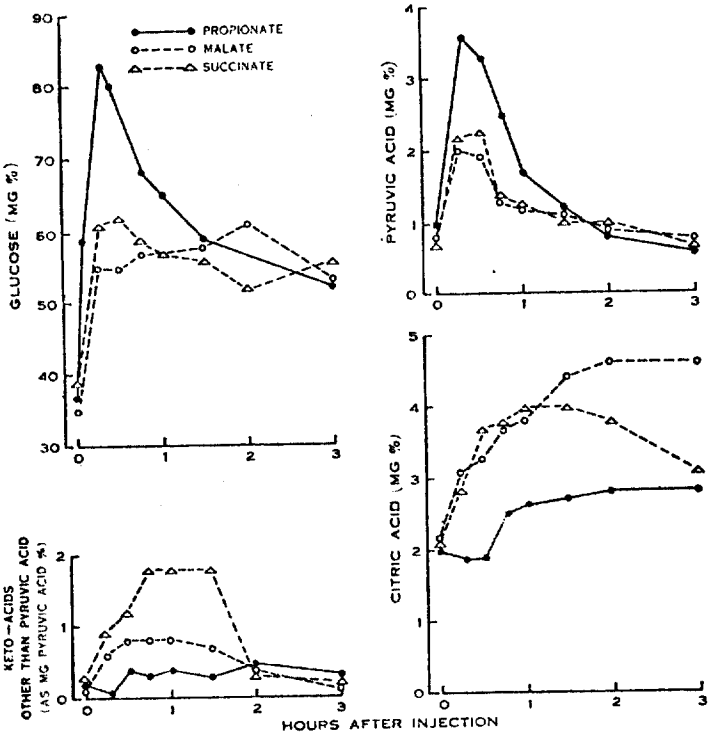


Fig. 5.—Changes in blood glucose, pyruvic acid, citric acid, and keto-acids other than pyruvic acid in three ewes after intravenous injection of propionate, succinate, or malate (2·25 m-moles per kg body weight).

Changes in blood pyruvic acid paralleled changes in blood glucose, particularly after propionate injection; ketones declined in two experiments. But, in the two experiments in which total keto-acids were also determined, the level of keto-acids other than pyruvic acid increased markedly after injection of succinate, but not after that of propionate (Fig. 4).

Experiment 2

The metabolic effects of propionate, succinate, and malate were next compared in experiments on three ewes. Total keto-acids and citric acid were determined in all experiments (Fig. 5).

(c) *Propionate, Succinate, and Malate Injections into Fed Non-Pregnant Ewes*

Experiment 1

The effects of injected propionate and succinate on blood glucose, pyruvic acid, total keto-acid, and ketone levels in fed, non-pregnant ewes were studied in "reversal" experiments on two ewes on the same two days (Fig. 4). These ewes were accustomed to this type of experimental procedure; saline injection into other ewes of the group was known to have no significant effect on blood glucose, pyruvic acid, and ketones (Fig. 6).

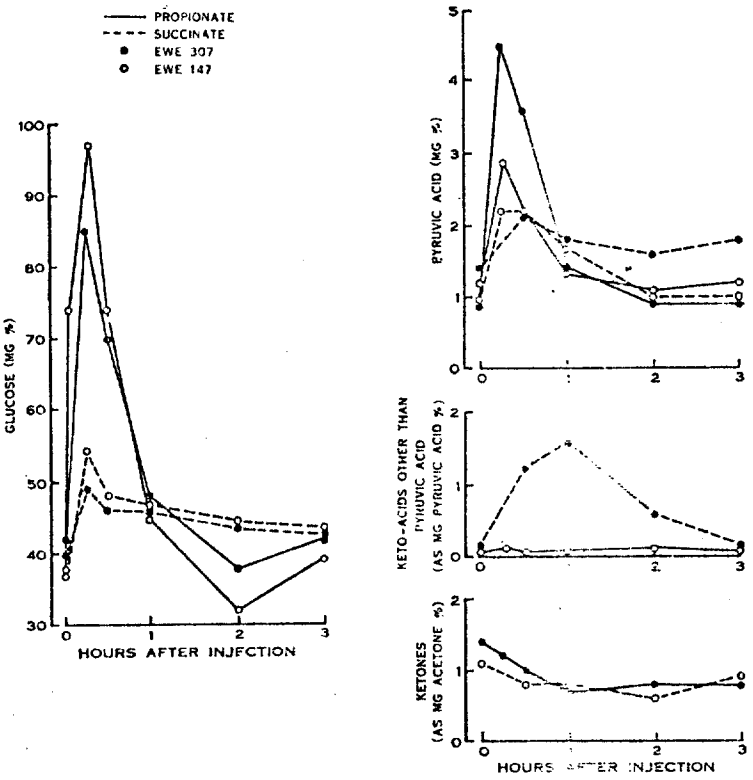
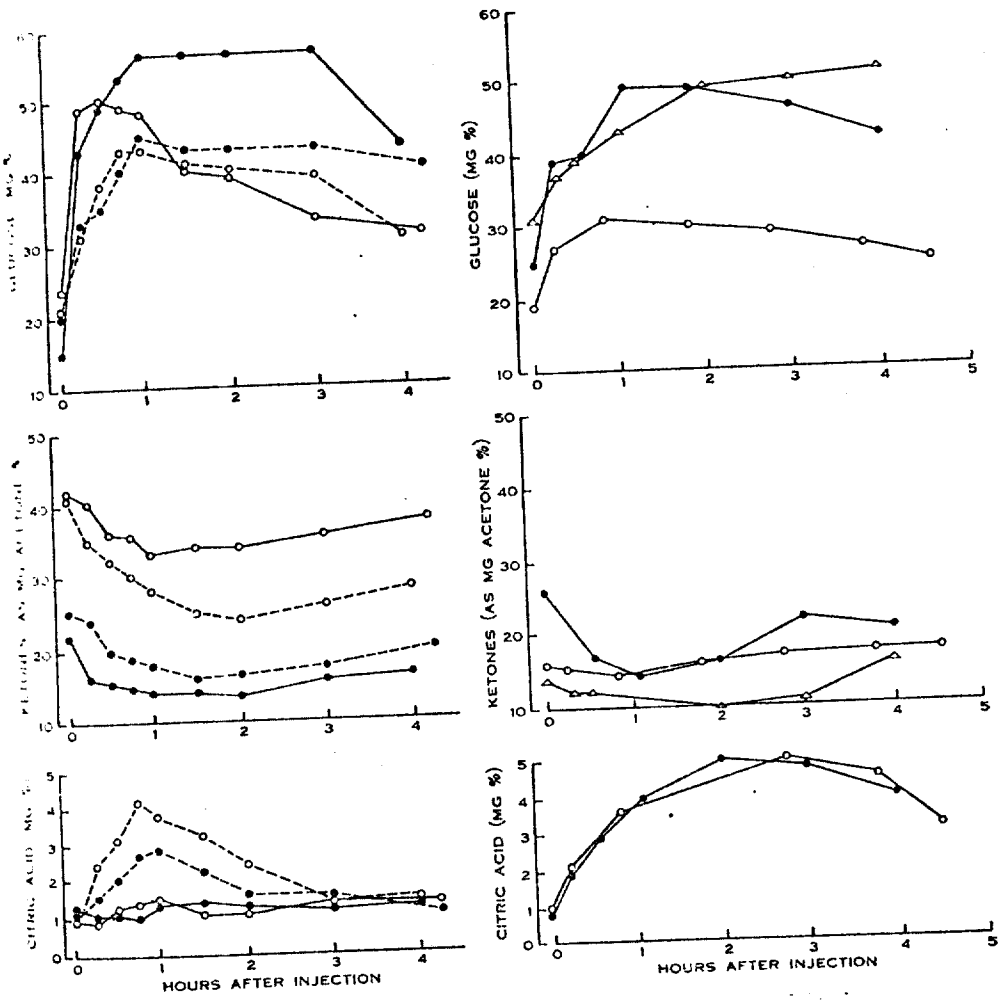


Fig. 4.—Changes in blood glucose, pyruvic acid, keto-acids other than pyruvic acid, and ketone bodies after intravenous injection of propionate and succinate (2 m-moles per kg body weight).

In contrast to the fasted pregnant ewes in which blood V.F.A. levels were 34 and 42 mg % 15 min after propionate injection (Fig. 1), V.F.A. levels had already returned to 10 mg % in the non-pregnant ewes at this time; the removal of injected propionate was virtually complete within 30 min. Acetate injections at the same dose rate and into the same sheep showed clearly that propionate was removed more rapidly than acetate; this confirms the observations of Jarrett, Potter, and Filsell (1952).

The blood citrate level increased markedly after malate injection in the two experiments in which it was measured (Fig. 3).



(2) (3)

Fig. 2.—Changes in blood glucose, ketones, and citric acid in fasted pregnant ewes after intravenous injection of propionate or succinate (2·25 m-moles per kg body weight). All experiments were done on two successive days.

●——● Ewe 25; propionate injection after 2 days of fasting.
●- - - -● Ewe 25; succinate injection after 3 days of fasting.
○——○ Ewe 806; propionate injection after 3 days of fasting.
○- - - -○ Ewe 806; succinate injection after 2 days of fasting.

Fig. 3.—Changes in blood glucose, ketones, and citric acid in fasted pregnant ewes after intravenous injection of malate (2·25 m-moles per kg body weight). All experiments were done on the same day after 3 days of fasting. ● Ewe 763; ○ ewe 764; △ ewe 776.

after succinate injection, to levels near normal; changes in ketones were similar to those after propionate injection (Fig. 2). However, whereas increases in blood citric acid after propionate injection were small, large increases followed succinate injection (Fig. 2).

Three experiments were carried out in which the effects of intravenous malate injection were studied (Fig. 3). The blood ketone level after injection in one ewe (763) was similar to that in ewe 25, previously given succinate (Fig. 1); changes in blood glucose and ketones were also similar. In general, the degree to which ketone

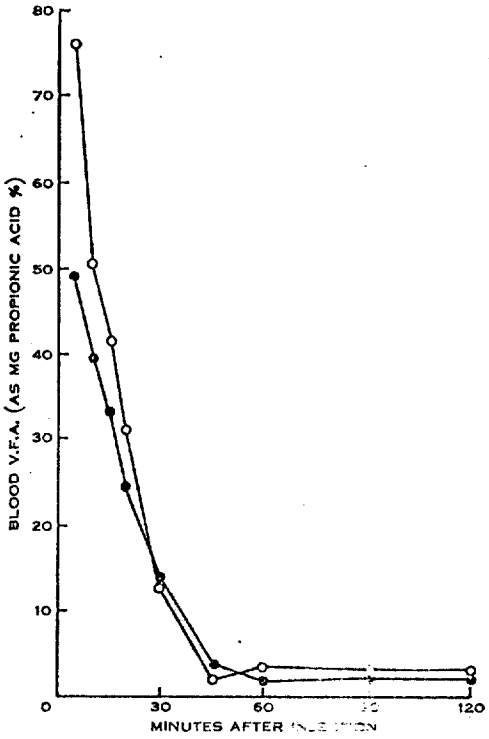


Fig. 1.—Disappearance of propionate injected intravenously into fasted pregnant ewes (2·25 m-moles per kg body weight). ● Ewe 25; ○ ewe 806 (see Fig. 2).

levels declined after succinate or malate injection depended on the pre-injection ketone level; the greatest decline occurred following succinate injection into ewe 806, which showed the highest ketone level (Fig. 2); the least decline was in ewes 764 and 776 (Fig. 3) after malate injection. However, it should be noted that no appreciable change in ketone level occurred in ewe 764 after malate injection and that this lack of response was associated with only a small increase in blood glucose. As the rate of removal of injected glucose appears to depend on the number of foetuses (Reid 1960*a*), it may be significant that this ewe was the only one in the experiments which carried twins.

Pronounced increases in both α-oxoglutaric and citric acids followed malate and succinate injections, but no significant changes occurred after propionate. No consistent changes occurred in oxalacetic acid levels.

Ketones showed a tendency to decrease but pre-injection levels were very low. In two fasted, non-pregnant ewes, ketones decreased from 6·7 and 8·3 mg % to 2·0 and 3·0 mg % respectively following succinate injection.

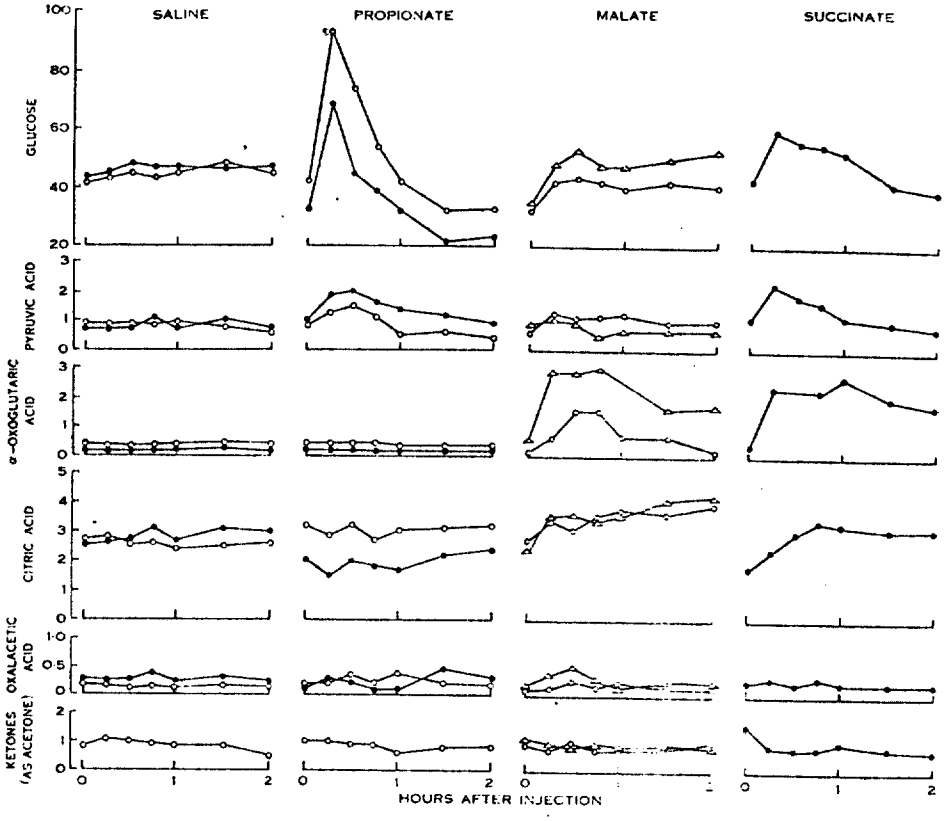


Fig. 8.—Changes in blood glucose, pyruvic acid, α-oxoglutaric acid, citric acid, oxalacetic acid, and ketones after intravenous injection of saline, propionate, malate, or succinate. ● Ewe 76; ○ ewe 25; △ ewe 99. Levels of blood constituents are in milligrams per 100 ml.

IV. Discussion

The mean blood pyruvic acid level of 1·0 mg % recorded here in fed, non-pregnant ewes compares with previously recorded values of 0·7 mg % for sheep (Jarrett and Potter 1954) and 1·0 mg % for man (Miller *et al.* 1952). Heyndrickx and Peeters (1960) recorded a mean value of 0·7 mg % in cattle plasma. These values were obtained by the method of Friedemann and Haugen (1943); the estimation of pyruvic acid after chromatographic separation on paper gives lower values of 0·55 mg % in cattle (Bach and Hibbitt 1959) and 0·3 mg % in man (Cavallini, Frontali, and Toschi 1949).

There appear to be no previous data on blood levels of citric, oxalacetic, and α-oxoglutaric acids in sheep. Our mean citric acid values of 3·0 and 3·4 mg % in fed non-pregnant and pregnant ewes respectively compare with values of 4·4 mg % (Bach and Hibbitt 1959) and 2·6 mg % (Heyndrickx and Peeters 1960) in cattle and 1·9 mg % in man (Wolcott and Boyer 1948). Our mean α-oxoglutaric acid value of 0·38 mg % compares with 0·19 mg % (Bach and Hibbitt 1959) and 0·10 mg % (Heyndrickx and Peeters 1960) in cattle and 0·2 mg % in man (Cavallini, Frontali, and Toschi 1949).

We have previously shown that blood citric acid declines in ewes undernourished in late pregnancy, and that citric acid levels in fed ewes are correlated with the degree of undernourishment, as indicated by the level of blood glucose (Reid and Hogan 1959). Lowest levels of citric acid are reached in severely hypoglycaemic fasted pregnant ewes; values are also low in ewes with pregnancy toxaemia induced in the laboratory, in which blood glucose values are similar to those in fasted, pregnant ewes (Table 1). But normo- or hyperglycaemia is relatively common in field cases of pregnancy toxaemia (Reid 1960*c*); the mean blood glucose in field cases in which citric acid was also determined was in the normal range (Table 1). If, as previously suggested, blood citric acid levels reflect the level of tricarboxylic acid cycle activity within the tissues (Reid and Hogan 1959; Reid 1960*a*), the fact that blood citric acid in these field cases of pregnancy toxaemia is as low as in hypoglycaemic ewes supports the suggestion of a depression of glucose metabolism (Reid 1960*a*, 1960*c*, 1960*d*).

If the analogy between the ewe with pregnancy toxaemia and the diabetic animal is correct (Reid 1960*a*, 1960*c*, 1960*d*, 1960*e*), this depression is likely to occur at a level of metabolism above pyruvate; intravenous glucose injection is followed by a pronounced increase in blood pyruvic acid in normal man (Bueding and Goldfarb 1943; Amatuzio *et al.* 1952; Miller *et al.* 1952), but not in diabetic individuals (Amatuzio *et al.* 1952; Miller *et al.* 1952). Blood pyruvic acid also increases after intravenous glucose injection into normal sheep (Jarrett and Potter 1954; Reid, unpublished data), but no detectable increase occurred in three ewes with pregnancy toxaemia (Reid, unpublished data).

Bach and Hibbitt (1959) recorded high pyruvate and low citrate levels in cows with clinical ketosis; blood α-oxoglutarate levels were also high. They concluded that an interference may take place with certain reactions of the Krebs cycle. Unfortunately, they compared ketotic cows with normal, fed animals. It appears possible from results presented here that part, at least, of the differences observed are not specific to the clinically affected animal, but are a normal consequence of fasting. Low citric acid levels may reflect a lowered metabolic rate and a lowered rate of entry of acetyl-coenzyme A (acetyl-CoA) into the tricarboxylic acid cycle.

The ketolytic effect of succinate in human diabetics is very variable (Beatty and West 1951). But succinate and malate each consistently reduced blood ketone levels in fasted pregnant ewes (Figs. 2, 3). However, a sustained increase in blood glucose occurred in all experiments; glucose injection would have been expected to reduce blood ketones in these ewes (Reid 1960*a*). It is obviously impossible to

differentiate between direct effects of succinate and malate on ketone utilization after conversion to oxalacetic acid and indirect effects arising from increased blood glucose levels. The fact that the fall in ketones was usually greater than would be expected after glucose injection, although blood glucose levels were much lower (Reid 1960*a*), is suggestive, but not conclusive.

Ho and Reber (1957) found that blood ketones in fasted, pregnant ewes declined after the injection of oxalacetic acid, and concluded that "oxalacetate was the effective substance in reducing ketone bodies". This interpretation is scarcely justified, because they recorded sustained increases in blood glucose far greater, in some animals, than could possibly be accounted for by conversion of oxalacetate to glucose, and because they carried out no control experiments.

The data of experiments presented in this paper emphasize the importance of suitable training of animals for experiments in which changes in blood glucose are a feature. The propionate injection experiment in Figure 5 is presumably confounded by a blood glucose increase following sympathetic stimulation. The experiments on fasted pregnant ewes (Fig. 2) may likewise be confounded, with the additional complication that the blood glucose changes are presumably modified by the presence of the foetus (Reid 1960*a*), and possibly by insulin insufficiency (Reid 1960*d*). Although blood glucose increases as rapidly after adrenaline as after propionate injection, it appears unlikely that the experiments recorded in Figures 4 and 7 are similarly complicated. The blood glucose changes after saline injection indicate that the injection procedure had little effect; the rapidity with which the excess glucose was removed (in contrast to the experiments in Figures 2 and 5) does not support a contention that propionic acid itself stimulated adrenaline secretion.

Because the increases in blood glucose following succinate and malate injections are much less than after propionate, the confounding effects of sympathetic stimulation make interpretation more difficult. In the experiments on inadequately trained animals (Figs. 2, 5), blood glucose increased by 22–26 mg % and this increase tended to be maintained throughout the experiment. However, in the experiments on trained animals (Figs. 4, 7), glucose increased by only 7 mg % to a definite peak at 15–30 min after injection and then declined. The fact that glucose may be still above the pre-injection level at 2 hr (Fig. 8) probably has little significance. At the dose rates used, conversion of succinate or malate to glucose could have caused a temporary increase in blood glucose of the order of only 10 mg %.

Injected succinate and malate may be metabolized via at least three pathways, all of which involve oxalacetate as an intermediate: (i) conversion to phosphoenolpyruvate and thence to glucose; (ii) conversion to pyruvate and thence to acetyl-CoA; (iii) conversion to oxalacetate, followed by re-entry into the tricarboxylic acid cycle. The operation of these pathways would explain the effects of succinate and malate injection on blood levels of glucose and of pyruvic, α-oxoglutaric, and citric acids. Failure of accumulation of oxalacetate would then be due to the high lability of this metabolic intermediate, any tendency to accumulate being counteracted by rapid decarboxylation to pyruvate. Previous experiments have shown that intravenous injection of succinate and malate increases urinary excretion of citrate

and α-oxoglutarate in rats and rabbits (Orten and Smith 1937; Krebs, Salvin, and Johnson 1938; Nordmann *et al.* 1955).

Likewise, malonate injection increases urinary excretion of citrate and α-oxoglutarate (Orten and Smith 1937; Krebs, Salvin, and Johnson 1938; Lee and Lifson 1951). Blood and tissue citrate levels are increased by fluoroacetate injection (Buffa and Peters 1950; Potter, Busch, and Bothwell 1951). In the only studies carried out on ruminants, oral fluoroacetate increased blood citrate levels (Jarrett and Packham 1956). The possibility that injected succinate and malate would also block the tricarboxylic acid cycle, leading to accumulation of citrate and α-oxoglutarate, but, again, not of oxalacetate, must also be considered. Such an effect might influence the response of blood ketones in fasted, pregnant ewes; acetyl-CoA oxidation might initially be stimulated, but later blocked by accumulating cycle intermediates.

However, the *precise* metabolic significance of the data obtained following succinate and malate injection is by no means clear. For example, the activity of isocitric dehydrogenase in the extra-mitochondrial region of the cell is very high. Accumulating citric acid may be released from mitochondria with the subsequent formation of α-oxoglutarate by a reaction outside the tricarboxylic acid cycle. The concentrations of citric and of α-oxoglutaric acids in blood may thus reflect an interaction between enzyme systems of different cell compartments and not simply the activity of the tricarboxylic acid cycle as such.

The present experiments, on the intact animal, in which the metabolic changes following propionate injection are so different from those following the injection of succinate or malate, do not appear to support the hypothesis that propionate is metabolized via succinate according to the scheme proposed by Flavin, Ortiz, and Ochoa (1955). However, it is clear that the metabolic system is so complex that such a conclusion would be unwarranted; the measured changes in blood levels of metabolites will reflect the interplay of all the different tissues of the body. Different tissues differ in their ability to activate and oxidize different substrates; injected propionate may be metabolized largely in liver whereas injected succinate and malate may be metabolized largely in extra-hepatic tissues. Differential permeability of cellular and subcellular membranes associated with differences in the physicochemical properties of the injected compounds may markedly influence the results observed.

Finally, it is by no means clear whether the pronounced increase in blood glucose following propionate injection is actually a result of direct conversion of propionic acid. The rate of increase of blood glucose is so very rapid (Fig. 4) that this may seem unlikely. On the other hand, injected propionate disappears very rapidly indeed in non-pregnant ewes, the maximum glucose level after injection coincides with or precedes the virtual disappearance of propionate, and the subsequent disappearance of glucose follows the pattern of the normal glucose tolerance curve. These observations are also consistent with the suggestion that the increase in blood glucose is due to a "sparing" effect of injected propionate on glucose oxidation.

It must be concluded that the experimental approach used in the injection experiments reported here has but limited application; the data merely confirm that the tricarboxylic acid cycle operates in sheep tissues.

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key-kidney antigen and to Fortner-tumor antigen in 2 weeks, indicating that development in hamsters of antibody to LCM is unrelated to the development of tumors.

No evidence of LCM has been observed in any hamsters bearing the Fortner fibrosarcomas. Newborn hamsters inoculated with cell transplants shortly after birth develop visible tumors in 7 to 10 days; the tumors enlarge rapidly and most animals die with massive tumors (25- to 50-mm) in 14 to 31 days. Weanlings develop palpable tumors within 10 to 12 days after receiving implants; none died after 2 months of progressive growth of tumors.

The data indicate that the Fortner fibrosarcoma No. 2 is contaminated with LCM virus and that presence of this virus in tumor transplants is responsible for the antibody response observed in tumor-bearing hamsters. Preliminary studies reveal that serum and organs (brains, lungs, liver, spleen, and kidney) from tumor-bearing hamsters that received implants during the first 24 hours of life also contain LCM virus. Consequently transplantation of tumor fragments from hamster to hamster results in a generalized infection and is simply a mode of inoculating virus. Development of high-titer complement-fixing antibody in weanling hamsters within 2 weeks of inoculation with cell-free tumor extracts corroborates this point.

Weanling hamsters bearing transplanted tumors induced by the Schmidt-Ruppin strain of Rous-sarcoma virus and an avian adenovirus–like agent (chicken-embryo lethal-orphan virus, CELO) (*10*) were housed in the same room with the animals bearing Fortner-fibrosarcoma No. 2 tumors. A number of the weanlings developed LCM-antibody titers of 1:80 or greater. Both the Rous and the CELO tumors were known to be free of LCM virus in earlier passages. One CELO-tumor antigen reacted at a 1:32 dilution with specific hamster antiserum to LCM. Also a number of hamsters bearing various primary or transplanted tumors (free of LCM in earlier passages), and housed in nearby facilities serviced by the same personnel, have developed complement-fixing antibody to LCM. Such data suggest that infected hamsters shed LCM virus and that a colony of susceptible hamsters may become contaminated with LCM by exposure to fomites.

Several weeks after the hamsters bearing Fortner-fibrosarcoma No. 2 tumors were introduced into the animal colony, personnel working with these animals developed influenza-like illnesses and serological evidence of LCM infection. This is further evidence of environmental contamination and indicates hazards that may attend work with transplantable rodent tumors, even when such transplanted tumors are derived from spontaneous or chemically induced neoplasms. The Fortner fibrosarcoma No. 2 had been contaminated by LCM while it was carried in certain other laboratories working on LCM infection in mice. Tumor samples received from one such laboratory were free of LCM virus and antigen.

Sabin (*11*) has shown that serological reactions to isoantigens in transplanted tumors may resemble the virus-specific reactions described by Huebner *et al.* (*1*). Our results demonstrate that a contaminating virus also may be a source of misleading results. Awareness of possible contamination of hamster tumors by LCM is also important for the protection of personnel. Presence of LCM virus in tumors has one possibly useful aspect: since the titer of LCM-complement-fixing antigen is superior to that obtained in other systems, the tumor extracts may be useful as potent and cheap diagnostic reagents.

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18 August 1965

Rabbit Muscle Lactate Dehydrogenase 5; A Regulatory Enzyme

Abstract. *Lactate dehydrogenase isozyme 5 from rabbit skeletal muscle is activated by citrate, cis-aconitate, isocitrate, α-ketoglutarate, succinate, fumarate, malate, aspartate, and glutamate. In the presence of these activators the shape of the pyruvate saturation curve is changed from sigmoid to hyperbolic. Lactate dehydrogenase isozyme 1 from rabbit heart gives a hyperbolic pyruvate saturation curve and is not activated by these compounds. Oxalacetate is a competitive inhibitor of both isozyme 5 and isozyme 1 but at low concentration it activates the former. These results indicate that lactate dehydrogenase isozyme 5 from rabbit skeletal muscle is an allosteric protein and a regulatory enzyme, while lactate dehydrogenase isozyme 1 from rabbit heart is apparently neither.*

A regulatory enzyme is one which takes part in the intracellular control of metabolic pathways. Evidence is accumulating that such enzymes have a number of characteristics in common; for example, (i) they are composed of subunits; (ii) the substrate saturation curves under certain conditions are sigmoid shaped; and (iii) they undergo conformational changes when exposed to "effectors" (*1*). The effectors may be activators or inhibitors and are bound to the enzyme at a site distinctly different from the substrate site. This type of molecular alteration has been termed an allosteric transition (*2*) and, as pointed out by Umbarger (*3*), is a special case of the "induced-fit" hypothesis of Koshland (*4*). Aspartate transcarbamylase is a classic example of a regulatory enzyme (*5–7*).

The subunit nature of various lactate dehydrogenases has been well documented (*8*). This report will present evidence that lactate dehydrogenase isozyme 5 (LDH 5) from rabbit skeletal muscle has a sigmoid-shaped substrate saturation curve which becomes hyperbolic in the presence of a number of effectors. The results suggest that this isozyme can be classified as a regulatory enzyme.

Twice recrystallized rabbit muscle LDH, which contains all five isozymes, was purchased from Worthington Biochemical Corporation. Pure LDH 5 was obtained from this prepa-

tion by chromatography on diethylaminoethyl Sephadex (*9*). Isozyme 1 was prepared from rabbit heart by the method of Wachsmuth and Pfleiderer (*9*). The bands were identified electrophoretically on polyacrylamide gels at *p*H 10.0. Assays were performed by measuring the rate of change of the 340 mμ absorption due to oxidation of reduced nicotinamide adenine dinucleotide (NADH) in a Beckman DU spectrophotometer at *p*H 7.4 and at 28° or 37°C. Protein determinations were based on a molar extinction coefficient for rabbit muscle LDH of 1.24×10^5 at 280 mμ (*10*). Enzyme dilutions were made with "Microcap" micropipettes obtained from Kensington Scientific Corporation.

The rate of the LDH 5–catalyzed conversion of pyruvate and NADH to lactate and nicotinamide adenine dinucleotide (NAD) is increased by seven citric acid cycle intermediates as well as by aspartic and glutamic acids which are directly converted to citric acid cycle intermediates. The activation is accompanied by a lowering of the apparent Michaelis constant (K_m) for pyruvate (Table 1). No significant change was noted in maximum velocity of conversion (V_{max}) values. No other amino acids had any effect on the reaction. These same compounds had little effect on the reaction catalyzed by LDH 1. Oxalacetate, the remaining citric acid cycle substrate, is an analogue of pyruvate and is a competitive inhibitor of LDH 5 at concentrations greater than $7 \times 10^{-4}M$. However, oxalacetate activates LDH 5 at lower concentrations. In contrast, oxalacetate, at all concentrations, inhibits LDH 1 (Fig. 1). Gerhart and Pardee (*6*) have made similar observations with aspartate transcarbamylase, using the aspartate analogues maleate and succinate. They have interpreted their data as being consistent with the idea that binding of a ligand at the substrate site at low substrate concentration, whether substrate or substrate analogue, brings about a molecular alteration leading to exposure of new substrate sites and to increased enzyme activity. As the substrate concentration is increased, the analogues compete for the more available substrate sites, and the result is decreased enzyme activity. My results thus suggested that the isozyme was capable of undergoing a conformational change and that the activators of LDH 5 were binding at an effector site.

Figure 2 is a pyruvate saturation curve and reveals that the curve for LDH 5 alone is slightly but definitely sigmoid shaped, whereas the curve in the presence of one of the activators, in this case *cis*-aconitate, is hyperbolic. The figure also shows that the curve for LDH 1 is hyperbolic.

Table 1. Effect of LDH 5 activators on apparent K_m for pyruvate. Initial reaction velocities were determined in 2-ml reaction mixtures containing $2.24 \times 10^{-5}M$ NADH; pyruvate concentration varied between $1 \times 10^{-5}M$ and $5 \times 10^{-4}M$; $1.5 \times 10^{-3}M$ activator; $20 \times 10^{-11}M$ LDH 5; $0.05M$ sodium phosphate buffer; *p*H 7.4 at 37°C. The K_m value were determined by the Lineweaver-Burk method with a least-squares program in an IBM 1620 computer to obtain the slopes and intercepts.

Compound added	$10^{-4}\ K_m$
Aspartate	1.29
α-Ketoglutarate	2.11
Succinate	1.46
Glutamate	2.46
Fumarate	1.35
cis-Aconitate	4.40
Malate	1.12
Isocitrate	3.31
Citrate	1.20
None	9.60

The other activators gave curves similar to that shown for LDH 5 and *cis*-aconitate, that is, their presence in the assay mixture resulted in a change in the curve from sigmoid to hyperbolic. These results further suggested that LDH 5 was capable of undergoing an allosteric transition. Gerhart and Pardee (*5*) found that they could destroy the allosteric site for cytidine triphosphate on aspartate transcarbamylase, without affecting the catalytic activity, by heating the enzyme at 60°C for 4 minutes. Heating LDH 5 for 3 minutes at 60°C completely destroyed the catalytic activity, but heating at 40°C for 3 minutes resulted in a desensitization of the enzyme toward the activators, while the catalytic activity remained unchanged (Fig. 3). Isozyme 1 is stable when heated at 60°C for 3 minutes. The assays for the data presented in Fig. 3 were carried out at 28°C, where the sigmoid shape of the LDH 5 substrate saturation curve is not as pronounced as it is at 37°C. The enzyme LDH 5 becomes desensitized toward the activators by standing in ice for 3 hours at a concentration of $4 \times 10^{-7}M$, while at a concentration of $5 \times 10^{-5}M$ (7 mg/ml) the enzyme retains full catalytic activity but is desensitized in about 2 weeks even in the cold room. Thus, rabbit muscle LDH 5 appears to be an allosteric protein while rabbit heart LDH 1 most likely is not.

Kaplan and his group (*11*) have

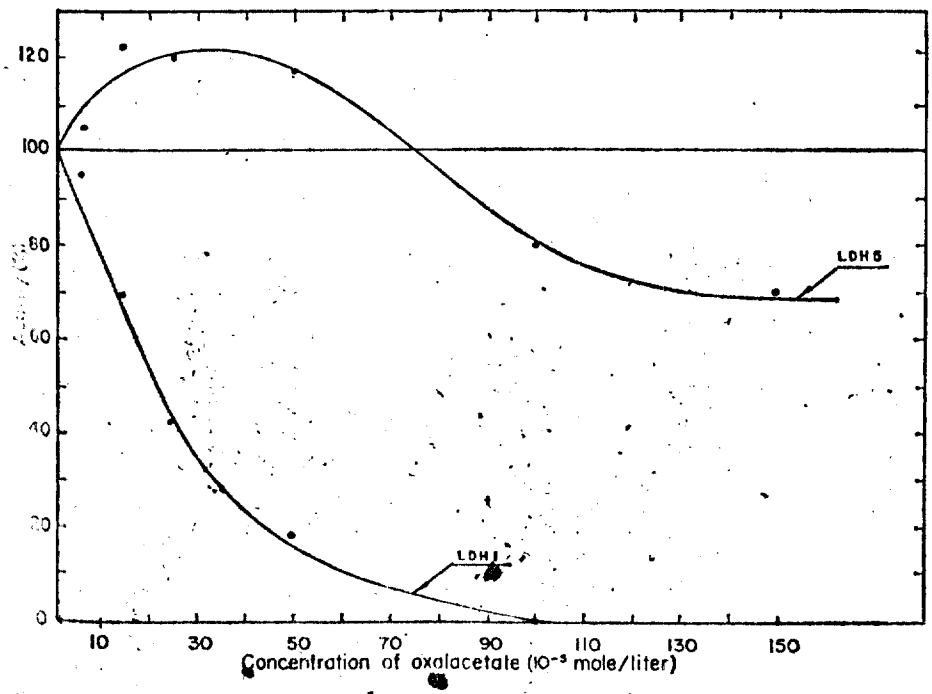

Fig. 1. Effect of oxalacetate on LDH activity. Assays were performed in a volume of 2 ml containing $2.24 \times 10^{-5}M$ NADH in $0.05M$ sodium phosphate buffer, *p*H 7.4 at 37°C. For LDH 5 the pyruvate concentration was $6.7 \times 10^{-4}M$, which was 19 percent of the saturating concentration. For LDH 1 the pyruvate concentration was $2.2 \times 10^{-4}M$, which was 35 percent of the saturating concentration. Enzyme concentrations were, for LDH 5, $20 \times 10^{-11}M$; for LDH 1, $7 \times 10^{-11}M$. Oxalacetate concentrations were as indicated.

previously proposed that LDH 5 and LDH 1 have significantly different functional roles. Their proposal was based on the fact that pyruvate concentrations which are subsaturating for LDH 5 are drastically inhibiting for LDH 1. The work here reported supports this proposal. Isozyme 5 is by far the most prevalent isozyme in skeletal muscle. It is a well-known fact that lactate accumulates in these muscles during violent exercise, when the oxygen supply becomes limiting. It is no doubt true that under these conditions the lactate accumulation may be due in part to increased NADH levels, but the activation of LDH 5 by the citric acid cycle substrates is probably a contributing factor. On the other hand, LDH 1 is the major molecular form in cardiac muscle, which fact, in view of the present observations, is not unexpected. A constant supply of energy is required by the heart, and it is furnished mainly by the adenosine triphosphate produced as a result of the oxidation of citric acid cycle substrates. In the heart these substrates do not feed back and limit their own concentration by activating LDH as they do in skeletal muscle.

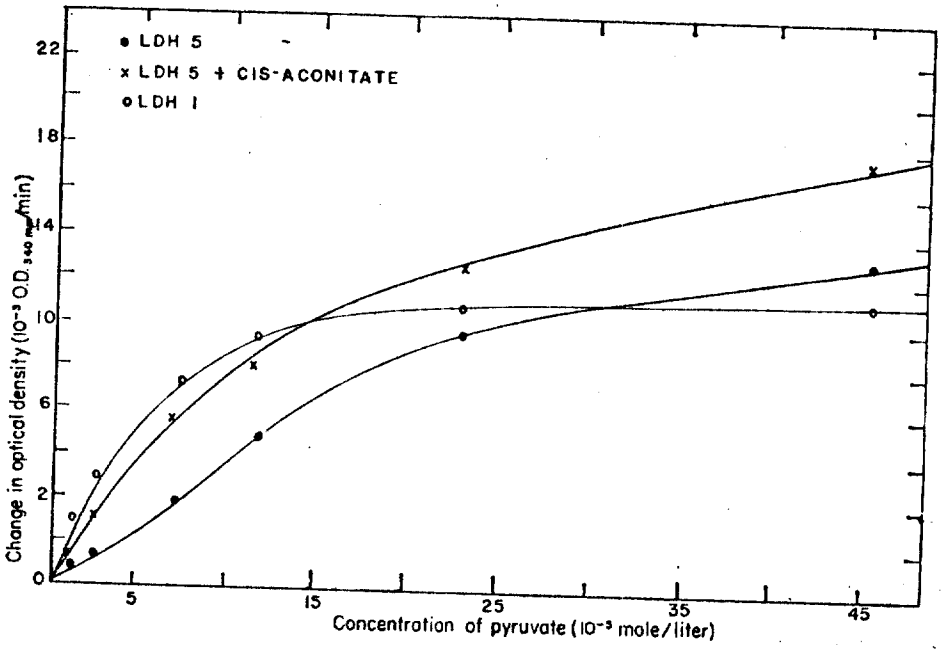

Fig. 2. Effect of activators on LDH 5 kinetics. The reaction mixtures were contained in 2 ml; $2.24 \times 10^{-5}M$ NADH and $1.5 \times 10^{-3}M$ *cis*-aconitate where used (see Table 1 for other activators which gave similar results); pyruvate concentrations as indicated; LDH 5, $20 \times 10^{-11}M$; LDH 1, $7 \times 10^{-11}M$; $0.05M$ sodium phosphate buffer, *p*H 7.4. Reactions were run at 37°C.

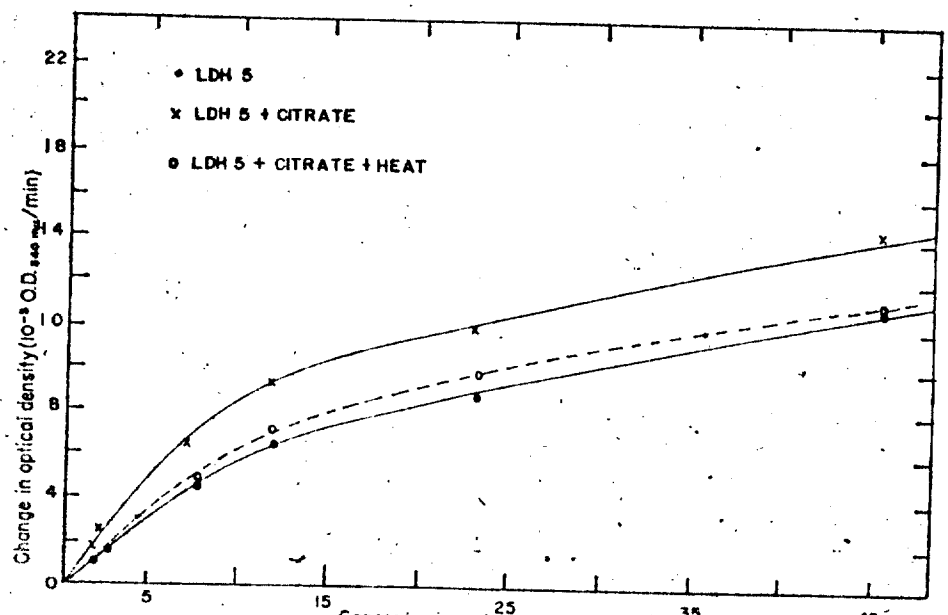

Fig. 3. Effect of heating on LDH 5 kinetics. The reaction mixtures were contained in 2 ml; $2.24 \times 10^{-5}M$ NADH and $1.5 \times 10^{-3}M$ citrate where used; pyruvate concentration as indicated; $0.05M$ sodium phosphate buffer, *p*H 7.4; LDH 5, $20 \times 10^{-11}M$. Reactions were run at 28°C. In the heated samples the enzyme at $4.2 \times 10^{-7}M$ was heated in a water bath at 40°C for 3 minutes, then diluted for the assay in the presence or absence of citrate. The assay in the absence of citrate gave the same curve as the unheated enzyme.

Another metabolic consequence of the LDH 5 behavior here reported would be a marked advantage of anaerobic glycolysis over aerobic respiration, since any activation of LDH would result in less pyruvate being available for conversion to acetylcoenzyme A and more NAD being available to spark glycolysis through the reaction catalyzed by triose phosphate dehydrogenase. Thus, when the citric acid cycle substrates act to reduce the concentration of a substance which is one of their precursors, they are in effect controlling their own concentrations, a simple case of feedback control by an "end product" of a biosynthetic pathway.

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Gluconeogenesis in the Kidney Cortex

FLOW OF MALATE BETWEEN COMPARTMENTS

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1. Kidney-cortex slices from starved rats were incubated with L-[U-^{14}C]lactate or L-[U-^{14}C]malate plus unlabelled acetate and the specific radioactivity of the glucose formed was determined. In parallel experiments the specific radioactivity of the glucose formed from [1-^{14}C]acetate plus unlabelled L-lactate and L-malate was determined. 2. By analytical methods the major products formed from the substrates were measured. The glucose formed was purified by paper chromatography for determination of specific radioactivity. 3. The specific radioactivity of the glucose formed from L-[U-^{14}C]lactate agrees with predictions of a model based on interaction of the gluconeogenic and the oxidative pathways. 4. The specific radioactivity of the glucose formed from L-[U-^{14}C]malate agrees with the predicted value if rapid malate exchange between the cytosol and mitochondria is assumed. 5. The rate of malate exchange between compartments was estimated to be rapid and at least several times the rate of glucose formation. 6. The specific radioactivity of the glucose formed from [1-^{14}C]acetate plus unlabelled L-lactate or L-malate agrees with the predictions from the model, again assuming rapid malate exchange between compartments. 7. Malate exchange between compartments together with reversible malate dehydrogenase activity in the mitochondria and cytosol also tends to equilibrate isotopically the NADH pool in these compartments. ^{3}H from compounds such as L-[2-^{3}H]lactate, which form NAD^{3}H in the cytosol, appears in part in water; and ^{3}H from DL-β-hydroxy[3-^{3}H]butyrate, which forms NAD^{3}H in the mitochondria, appears in part in glucose, largely on C-4.

In gluconeogenesis from pyruvate, it has been proposed that malate flows out of the mitochondria to provide the required cytoplasmic reducing equivalents (Lardy, Paetkau & Walter, 1965; Krebs, Gascoyne & Notton, 1967). The preceding paper also supports this hypothesis (Rognstad & Katz, 1970). Experiments by Haslam & Krebs (1968) with isolated mitochondria have suggested inflow of malate to require energy. To investigate the problem of malate flow between the mitochondria and the cytosol in the intact cell, we determined the specific radioactivity of the glucose formed when kidney-cortex slices were incubated with L-[U-^{14}C]malate plus unlabelled acetate. From the dilution in specific radioactivity of the glucose formed compared with that of the substrate, we have concluded that malate exchange between compartments is rapid, at least under conditions that permit active gluconeogenesis.

EXPERIMENTAL

All enzymes were obtained from either Calbiochem, Los Angeles, Calif., U.S.A., or Sigma Chemical Co., St Louis, Mo., U.S.A. L-[U-^{14}C]Lactate, [1-^{14}C]acetate and [^{3}H]$NaBH_4$ were from New England Nuclear Corp., Boston, Mass., U.S.A. L-[U-^{14}C]Malate and [2-^{14}C]-pyruvate were from Amersham/Searle, Des Plaines, Ill., U.S.A. L-[2-^{14}C]Lactate was prepared by reduction of [2-^{14}C]pyruvate with NADH in the presence of lactate dehydrogenase. DL-[2-^{3}H]Malate, DL-[2-^{3}H]lactate and DL-β-hydroxy[3-^{3}H]butyrate were prepared by reduction with [^{3}H]$NaBH_4$ of, respectively, oxaloacetate, pyruvate and acetoacetate (Lowenstein, 1963). L-[2-^{3}H]Lactate was synthesized as follows: DL-[2-^{3}H]malate (3μmol), NAD^+ (15μmol), acetyl phosphate (10μmol), CoA (1μmol), triethanolamine–HCl buffer, pH 8.0 (300μmol), malate dehydrogenase (5 units), phosphotransacetylase (3 units; EC 2.3.1.8) and citrate synthase (3 units) in a final volume of 3ml were incubated in a cuvette and NAD^{3}H formation was followed at 340nm. Pyruvate (20μmol) and lactate dehydrogenase (10 units) were then added and L-[2-^{3}H]-lactate formation was followed by the decrease in E_{340}. The reaction mixture was put on a column (1cm × 11cm) of Dowex1 (X8; 100–200 mesh acetate form) (Calbiochem). L-[2-^{3}H]Lactate was eluted with 1M-formic acid, evaporated to dryness and further purified by paper chromatography with ethanol–aq. NH_3 (sp.gr. 0.88)–water (16:1:3, by vol.). L-[2-^{3}H]Malate was synthesized as follows: DL-[2-^{3}H]lactate (2μmol), NAD^+ (10μmol), L-glutamate (50μmol), glycine–NaOH buffer, pH 9.5 (200μmol), lactate dehydrogenase (20 units) and glutamate–pyruvate

transaminase (8 units) were incubated in a 3ml volume and NAD^{3}H formation was followed at 340nm. Oxaloacetate (10μmol) and malate dehydrogenase were then added. The L-[2-^{3}H]malate formed was purified by a procedure similar to that used for L-[2-^{3}H]lactate.

Kidney-cortex slices were obtained from starved rats except where noted. The incubation procedure is given in the preceding paper (Rognstad & Katz, 1970). The incubation medium was that of Krebs, Hems & Gascoyne (1963), which contained no bicarbonate. At the end of the incubation, CO_2 was collected by injecting 0.3ml of 4M-NaOH (CO_2-free) into a hanging plastic well and acidifying the medium. After at least 2½h the well was removed and the contents were added to 4.7ml of CO_2-free water. A sample of this was taken for manometric CO_2 determination in a Warburg apparatus. The amount of glucose initially present in the slices was found never to exceed 0.1μmol. The initial L-lactate content also was less than 0.1μmol. Control flasks were also run containing buffer and tissue that were acidified at zero time and a 'CO_2 blank' was determined. This value never exceeded 1μmol, and the blank value was subtracted to give the CO_2 values in Table 1.

A sample (8ml) of the acidified medium (total vol. 10ml) was put through a column (1cm×10cm) of Dowex 50 (X8; 50–100 mesh; H^+ form) and a column (1cm×11cm) of Dowex 1 (X8; 100–200 mesh; acetate form), arranged in tandem, both of which were washed with water until 25ml of effluent was collected. This was evaporated to dryness and chromatographed on Whatman 3MM paper with butan-1-ol–acetic acid–water (4:1:2, by vol.). Glucose spots were located with X-ray film. The specific radioactivity of the purified glucose was measured, with glucose determination by the method of Slein (1965). All radioactivity counting was done with a Packard model 3375 liquid-scintillation counter.

Yields of ^{3}H in water were determined either by freeze-drying of the neutralized medium or, when yields were high, by the difference in ^{3}H radioactivity between identical samples of the neutralized medium with or without prior evaporation.

The location of ^{3}H in glucose was determined by an enzymic procedure. It is assumed that, as found by Hoberman & D'Adamo (1960), all ^{3}H was on either C-4 or C-6. Glucose was converted into 3-phosphoglycerate with purified glycolytic enzymes and the required cofactors in the presence of arsenate plus excess of acetaldehyde and alcohol dehydrogenase to oxidize the NADH formed (Schmidt, Genovese & Katz, 1970). The amount of NAD^+ added was less than 5% of the original glucose. Unlabelled glucose was later added to ensure turnover of the NADH. The radioactivity of samples of the medium was counted with and without prior evaporation. The difference in ^{3}H radioactivity measured gives the ^{3}H originally located on C-4 of glucose. Trials were run with authentic [4-^{3}H]glucose and [6-^{3}H]glucose to determine the validity of the procedure.

RESULTS AND DISCUSSION

When kidney-cortex slices from starved rats are incubated without any added substrate, a small amount of glucose is formed from endogenous substrates, generally less than 0.5μmol/2h per 125mg wet wt. However, a considerable amount of carbon dioxide is formed, corresponding to the oxidation of nearly 10μmol of acetyl-CoA/2h per 125mg. The main source of the acetyl-CoA is presumably lipid. If acetate is added, no additional glucose is formed, but there is usually some increase in carbon dioxide production. Acetate was used in nearly all the experiments described to maintain a relatively constant flow of acetyl-CoA to the tricarboxylic acid cycle, rather than depending on endogenous sources, which may vary between experiments. Addition of acetate also tends to diminish oxidation of pyruvate to acetyl-CoA. This is desirable because measurement of this flow is only approximate.

When lactate and acetate are used as substrates

Table 1. *Metabolism of* L-*lactate and* L-*malate by kidney-cortex slices*

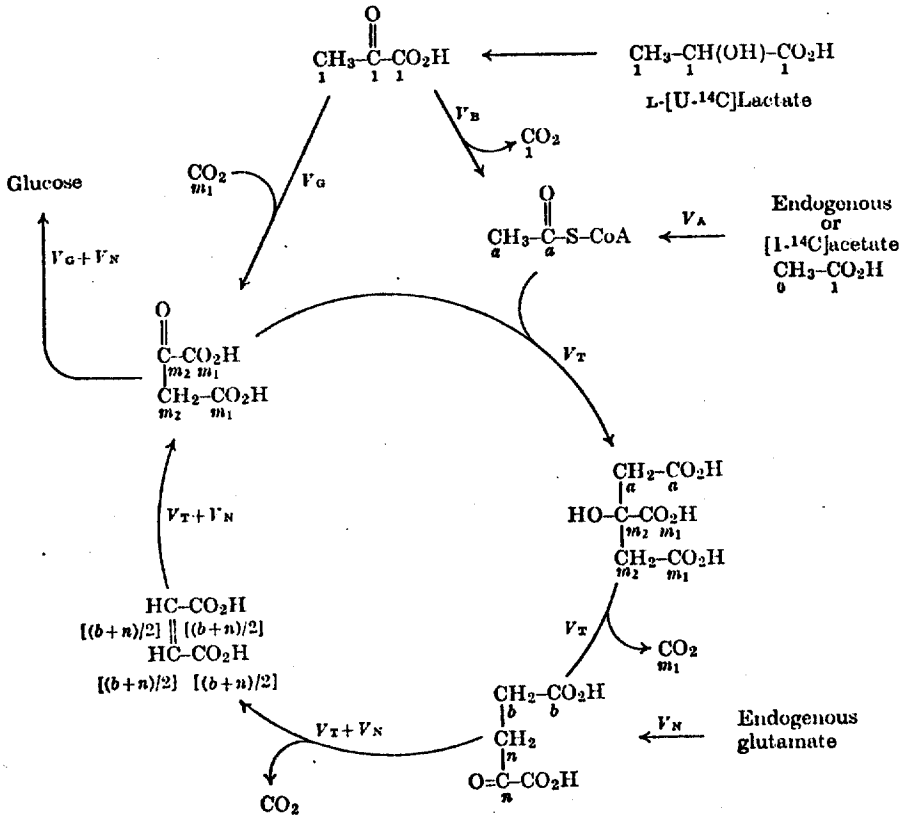
Kidney-cortex slices from starved rats were incubated for 2h at 37°C with substrates as shown plus 20μmol of acetate. Glucose formation without substrate was 0.4±0.1μmol/2h per 125mg wet wt. and has been subtracted to give the values in the table. Substrate oxidation is calculated from the amount of substrate used minus that converted into glucose, lactate and pyruvate. Results are expressed as μmoles/2h per 125mg wet wt. n.d., Not determined.

Expt. no.	Substrate	Amount added (μmol)	Substrate used	Substrate converted into glucose	Lactate formed	Pyruvate formed	Total CO_2 produced	Substrate oxidized
2	L-Lactate	16	8.7	7.6	—	0.5	37.0	0.6
	L-Malate	16	14.5	11.2	0.8	n.d.	50.4	2.5
3	L-Lactate	16	9.0	6.6	—	0.6	30.9	1.8
	L-Malate	16	13.5	11.6	0.8	n.d.	52.3	1.1
4	L-Lactate	8	7.2	6.6	—	0.1	36.3	0.5
	L-Malate	8	7.0	6.9	0.4	n.d.	40.5	0
6	L-Lactate	16	8.7	5.7	—	0.6	37.9	2.4
	L-Malate	16	11.1	8.5	0.6	0.1	43.7	1.9

most of the lactate consumed is converted into glucose, and only a small amount is oxidized to acetyl-CoA (Table 1). The flow of acetyl-CoA is estimated from the amount of lactate that disappears minus that converted into glucose and pyruvate. This neglects formation of other minor products. An independent method (R. Rognstad & J. Katz, unpublished work) using the pattern of ^{14}C labelling in C-1 and C-5 of glutamate isolated from experiments with L-[U-^{14}C]lactate or L-[2-^{14}C]-lactate has shown agreement with the method based on analysis.

If L-[U-^{14}C]lactate is used as substrate, the molar specific radioactivity of the glucose formed via a pathway that involved synthesis from two L-[U-^{14}C]lactate molecules without dilution of specific radioactivity naturally would be twice that of the original lactate. Such a pathway, e.g. via reversal of pyruvate kinase, is no longer considered of any physiological significance. As shown in Table 2, the molar specific radioactivity of the glucose formed in the experiment is even less than that of the lactate. The most likely reason for this dilution in specific radioactivity is found in the 'crossing-over' phenomenon (discussed by Krebs, Hems, Weidemann & Speake, 1966) in which labelled carbon from the gluconeogenic pathway mixes with unlabelled carbon of the oxidative pathway (tricarboxylic acid cycle) because mitochondrial oxaloacetate is an intermediate common to both pathways.

Scheme 1 shows a model of the metabolism of



Scheme 1. Metabolism of L-[U-^{14}C]lactate and unlabelled acetate in the kidney cortex. The small letters or numbers under each carbon atom represent molar specific radioactivities of these atoms. The rates, V, are molar rates. V_G is the rate of gluconeogenesis from added substrate; V_B is the rate of pyruvate dehydrogenase; V_T is the rate of the tricarboxylic acid cycle; V_A is the rate of formation of acetyl-CoA from added acetate and from endogenous sources; V_N is the rate of gluconeogenesis from endogenous glutamate. M_1 represents the specific radioactivity of the carboxyl carbon of the dicarboxylic acids, oxaloacetate, malate and fumarate, which are assumed to be in isotopic equilibrium. m_2 represents the specific radioactivity of the interior carbon atoms of these dicarboxylic acids. The lower dicarboxylic acid is used to show the specific radioactivity of the inflowing carbon from the forward tricarboxylic acid cycle. When L-[U-^{14}C]lactate is the substrate, the specific radioactivity of each carbon atom of lactate is set equal to 1. When [1-^{14}C]acetate is the substrate, the specific radioactivity of C-1 is set equal to 1.

Table 2. *Dilution in specific radioactivity of glucose formed from* L-[U-^{14}C]*lactate or* L-[U-^{14}C]*malate*

For experimental conditions, see Table 1. V_T is the rate of the tricarboxylic acid cycle; V_B is the rate of the pyruvate dehydrogenase reaction; V_N is the rate of formation of glucose from endogenous substrates; V_E is the rate of exchange of malate between mitochondria and cytosol. All of these are expressed relative to the rate of conversion of glucose, V_G, set equal to 1.0. V_B is estimated from the amount of substrate oxidized (Table 1). V_T is estimated from the amount of CO_2 formed in the experiment, measured manometrically, minus that formed in the pyruvate dehydrogenase reaction (V_B) and also, when malate is substrate, minus that produced from C-4 of malate utilized. $R_{exp.}$ is the dilution in specific radioactivity of the glucose formed and is defined by: $R_{exp.}$ = (sp. radioactivity of glucose)/2(sp. radioactivity of C-1, C-2 and C-3 of substrate). When L-[U-^{14}C]lactate is the substrate, $R_{theoret.}$ is calculated by:

$$R = \frac{(2V_G+V_B)(2V_G+5V_T+4V_N)+(V_G+V_B)(2V_G+V_T+2V_N)}{3(2V_G+V_T+2V_N)(V_G+2V_T+2V_N)} \quad \text{... (A1)}$$

When L-[U-^{14}C]malate is the substrate, $R_{theoret.}$ is calculated by:

$$R = \frac{2(VG+V_B)}{2V_G+V_B+V_T+2V_N} \quad \text{... (A4).}$$

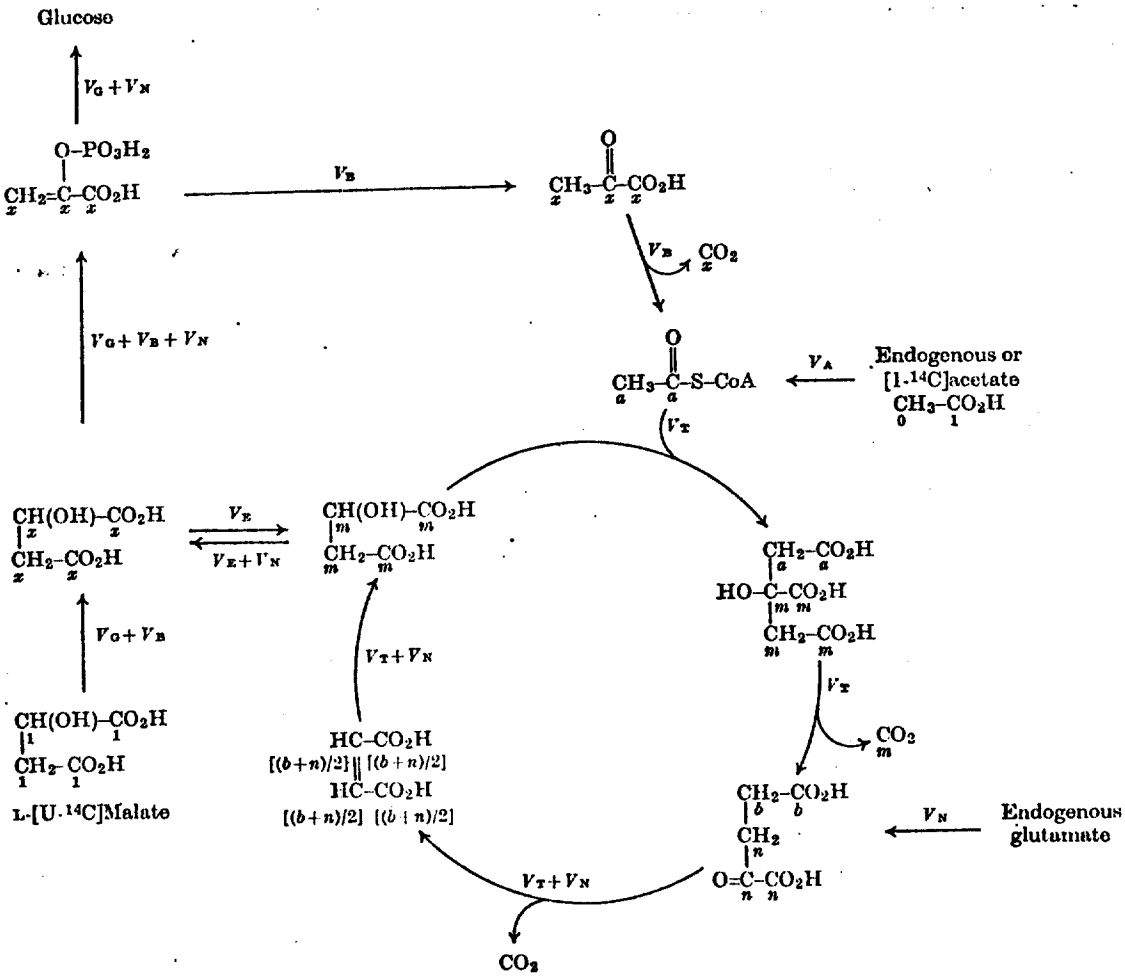
This equation assumes complete equilibration of intra- and extra-mitochondrial malate ($V_E = \infty$). V_E is calculated from:

$$V_E = \frac{(V_T+2V_N)(V_G+V_B)-R[(V_G+V_B+V_N)(V_T+2V_N)-V_NV_B]}{R(2V_G+V_B+2V_N+V_T)-2(V_G+V_B)} \quad \text{... (A3),}$$

by using the experimental value of R. The equations are derived in the Appendix.

Expt. no.	Substrate	Amount added (μmol)	Acetate (μmol)	Sp. radioactivity of substrate (c.p.m./μmol)	Sp. radioactivity of glucose (c.p.m./μmol)	Sp. radioactivity of CO_2 (c.p.m./μmol)	$R_{exp.}$	V_T	V_B	V_N	$R_{theoret.}$	V_E	m_t (c.p.m./μmol)
1	L-[U-^{14}C]Lactate	8	0	152000	149000	—	0.49	—	—	—	—	—	—
	L-[U-^{14}C]Malate	8	0	99500	89200	—	0.60	—	—	—	—	—	—
2	L-[U-^{14}C]Lactate	16	20	91700	80700	10900	0.44	2.20	0.07	0.12	0.44		11500
	L-[U-^{14}C]Malate	16	20	49400	48000	—	0.65	1.50	0.21	0.08	0.63	7.1	—
3	L-[U-^{14}C]Lactate	16	20	152000	153000	—	0.50	2.08	0.24	0.12	0.49	—	—
	L-[U-^{14}C]Malate	16	20	101000	92700	—	0.61	1.53	0.09	0.07	0.58	5.4	—
4	L-[U-^{14}C]Lactate	8	20	121000	104000	13400	0.43	2.40	0.07	0.12	0.42	—	14800
	L-[U-^{14}C]Malate	8	20	109000	79800	—	0.49	2.11	0	0.12	0.46	9.6	—
5	L-[U-^{14}C]Lactate	8	10	133000	110000	—	0.41	—	—	—	—	—	—
	L-[U-^{14}C]Malate	8	10	120000	84800	—	0.47	—	—	—	—	—	—
6	L-[U-^{14}C]Lactate	16	20	280000	252000	37600	0.45	2.73	0.37	0.15	0.45	—	37100
	L-[U-^{14}C]Malate	16	20	351000	329000	—	0.62	1.60	0.21	0.10	0.60	10.6	—

L-[U-^{14}C]lactate and unlabelled acetate in the kidney-cortex. The rates (V values) can all be estimated from the analytical results. There are a number of approximations involved in the model. The specific radioactivity of the carbon dioxide fixed in the pyruvate carboxylase reaction is set equal to the specific radioactivity of the carbon dioxide produced in the tricarboxylic acid cycle. This is strictly true only if V_B, the rate of the pyruvate dehydrogenase reaction, is zero. Mitochondrial oxaloacetate, malate and fumarate are assumed to be in complete isotopic equilibrium. This is a good approximation, as shown by Walter, Paetkau & Lardy (1966). Recycling of phosphoenolpyruvate to pyruvate is neglected. In section *A* of the Appendix is calculated, on the basis of the model, the expected dilution in specific radioactivity (denoted by R) of the glucose formed. R is defined as the ratio of the specific radioactivity of the glucose formed to twice the specific radioactivity of C-1, C-2 and C-3 of the substrate. Table 2 shows the comparison of the experimental values of R and the values of R calculated on the basis of Scheme 1. The agreement between experimental and theoretical values is good. In view of the approximations used, Scheme 1 does not necessarily represent the complete physiological situation, but it probably includes the major metabolic pathways involved. Thus the major cause of the dilution in specific radioactivity indeed appears to be the



Scheme 2. Metabolism of L-[U-^{14}C]malate and unlabelled acetate in the kidney cortex. x represents the specific radioactivity of each carbon atom of extramitochondrial malate, and m represents the specific radioactivity of each carbon atom of intramitochondrial malate. V_E represents the rate of exchange flow of malate between these compartments. When [1-^{14}C]acetate is the substrate, V_E is set equal to infinity, and $x = m$.

interaction of the gluconeogenic pathway and the tricarboxylic acid cycle.

One of the assumptions used in the model is that the specific radioactivity of the carbon dioxide fixed in the pyruvate carboxylase reaction was the same as that of the carboxyl carbon atoms of the dicarboxylic acids, or C-3 and C-4 of glucose. Table 2 shows that the values of m_1 (representing C-3 and C-4 of glucose), calculated from the equations of the Appendix and the experimental specific radioactivity of glucose, are quite close to the specific radioactivities of the carbon dioxide formed. Use of this assumption hence causes little error.

In gluconeogenesis from malate plus acetate, it is again found that most of the malate that disappears is converted into glucose, with a small amount being oxidized to carbon dioxide via acetyl-CoA (Table 1). Since the conversion of malate into glucose involves strictly a cytoplasmic pathway, it might be expected that the molar specific radioactivity of the glucose formed would be twice that of C-1, C-2 and C-3 of L-[U-^{14}C]malate, i.e. values for R of 1.0 might be expected. Actually the dilution in specific radioactivity approaches that found when L-[U-^{14}C]-lactate is converted into glucose (Table 2). The simplest explanation for the dilution in specific radioactivity would seem to be that of an exchange flow of malate between the cytosol and the mitochondria, in this way again producing a mixing of labelled carbon atoms from the gluconeogenic pathway with unlabelled carbon atoms from the tricarboxylic acid cycle.

Scheme 2 shows the model of the metabolism of L-[U-^{14}C]malate and unlabelled acetate by the kidney cortex. Separate pools of extramitochondrial and intramitochondrial malate are assumed, with the rate of exchange flow of malate between these pools denoted by V_E. In section B of the Appendix is calculated the expected dilution in glucose specific radioactivity ($R_{theoret.}$) for L-[U-^{14}C]-malate as substrate, when V_E is assumed to be infinite (eqn. A4) or when intermediate values of V_E are assumed (eqn. A2). It is seen (Table 2) that the values of $R_{theoret.}$ calculated on the basis of a single malate pool (i.e. $V_E = \infty$) are close to the experimental values of the dilution ($R_{exp.}$). If the experimental values of R are used to calculate V_E, from eqn. (A3), rates of 5–10 times the rate of glucose formation are found. Fig. 1 shows a plot of V_E versus R, calculated from eqn. (A2), for an example with typical values of V_T (rate of tricarboxylic acid cycle), V_B (rate of pyruvate dehydrogenase) and V_G (rate of gluconeogenesis from added substrate). At high rates of exchange the curve is rather flat and accurate determination of V_E is difficult in this region. As V_E approaches V_G, R becomes very sensitive to the rate of exchange. The values of V_E calculated in Table 2 can only be considered approximate, since these are in the region where R is insensitive to V_E. However, it should be safe to say that the exchange rate is rapid

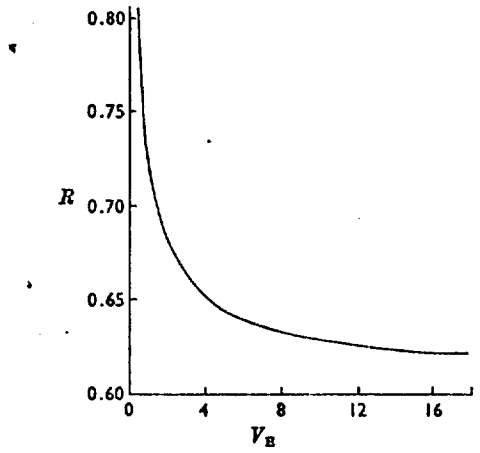


Fig. 1. Effect of the rate of malate exchange between mitochondria and cytosol on the dilution of the specific radioactivity of glucose formed from L-[U-^{14}C]malate. An example with typical values of the experimental rates is considered: $V_G = 1$; $V_T = 1.5$; $V_B = 0.1$. V_N is set equal to 0. Various values of V_E are assumed, and R is calculated for each of these values according to eqn. (A3).

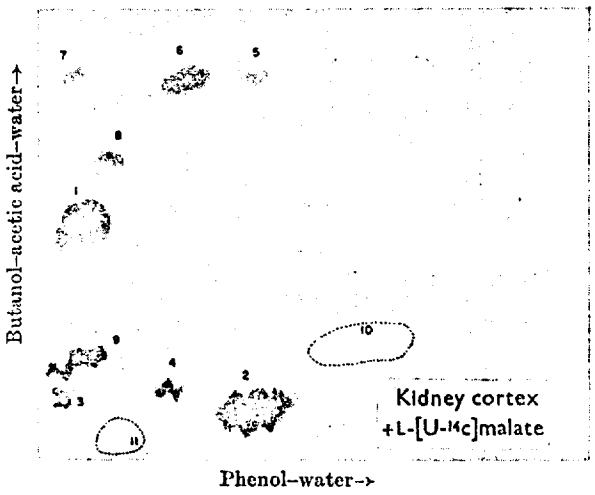


Fig. 2. Radioautograph of desalted medium from the incubation of L-[U-^{14}C]malate and unlabelled acetate with kidney-cortex slices. The numbers denote the following compounds: 1, malate; 2, glucose; 3, citrate plus isocitrate; 4, glutamate; 5, lactate; 6, succinate; 7, fumarate; 8, α-oxoglutarate (?); 9, unknown; 10, alanine; 11, aspartate. The method is that of Katz & Chaikoff (1954).

and at least several times the rate of glucose-synthesis.

In addition to the approximations used in setting up the models there are other considerations that also make an exact determination of the exchange rate difficult. When the medium from an incubation with L-[U-^{14}C]malate is electrolytically desalted and subjected to two-dimensional chromatography and the radioautographs are examined, there is radioactivity in several other compounds besides glucose and the substrate (Fig. 2). Succinate, glutamate, citrate and other intermediates become labelled. This may mean that exchange of label also occurred with the pre-existing pools of these various intermediates, in addition to the exchange with the large inflow of acetyl-CoA from endogenous sources. However, the amount of radioactivity in these compounds is less than one-tenth that in carbon dioxide and glucose, and neglect of this exchange should not cause a large error in the calculated exchange rate. Another factor to be considered is that a small proportion of cells other than cortex cells may be present, e.g. medulla cells, which oxidize acetate but form no glucose. This would mean that the dilution by unlabelled acetyl-CoA in the kidney-cortex is overestimated by the manometric carbon dioxide results. The two possibilities discussed in this paragraph would give opposite effects. Neglect of exchange with pre-existing pools of intermediates means that V_E is overestimated, whereas neglect of oxidation of acetate by non-cortex cells causes V_E to be underestimated.

In two experiments the specific radioactivity of the residual malate after incubation with L-[U-^{14}C]-malate was determined and found to be unchanged from the original. However, the specific radioactivity of the residual lactate from an incubation with L-[U-^{14}C]lactate was about 10% less than that of the original substrate. This may reflect some dilution of the lactate pool due to recycling of phosphoenolpyruvate to pyruvate.

A corollary of the exchange is illustrated by the results of Table 3. Here, labelled [1-^{14}C]acetate and unlabelled L-lactate or L-malate were incubated with kidney-cortex slices and the specific radioactivity of the glucose was again determined. In this case, labelled carbon from the oxidative pathway mixes with unlabelled carbon from the gluconeogenic pathway either by exchange in the case of malate or probably by a convergence of the two pathways in the case of lactate. Again the specific radioactivity of the glucose formed agrees reasonably well with predictions based on the model shown in Scheme 1 and described in Section *C* of the Appendix, assuming only a single malate pool.

The consequences of malate exchange between compartments include effects on the specific radioactivity of the NAD^{3}H produced from tritiated substrates that form NAD^{3}H in either the cytosol or the mitochondria. Hoberman & D'Adamo (1960) were the first to use L[2-^{2}H]- and L-[2-^{3}H] lactate as tracers for reductive hydrogen in gluconeogenesis. In gluconeogenesis from L-[2-^{3}H]lactate, the molar ^{3}H specific radioactivity of the glucose formed, in the absence of isotope discrimination or exchange reactions with water, should be about 1.0. The NAD^{3}H formed in the lactate dehydrogenase reaction reduces 1,3-diphosphoglycerate to [1-^{3}H]-glyceraldehyde phosphate, which isomerizes to dihydroxy[1-^{3}H]acetone phosphate. When the triose phosphates react via aldolase, the ^{3}H on dihydroxyacetone phosphate is lost to water (Rose & Rieder, 1955), and only one ^{3}H atom (from [1-^{3}H]glyceraldehyde phosphate) is incorporated into glucose, on C-4. However, if malate moves rapidly into and out of the mitochondria, and if the malate dehydrogenases in the cytosol and mitochondria both operate reversibly, isotopic mixing of the cytoplasmic and mitochondrial NADH

Table 3. *Molar specific radioactivity of glucose formed from* [1-^{14}C]*acetate plus unlabelled* L-*lactate or* L-*malate*

For experimental conditions, see Table 1. The incubation medium contained 16 μmol of unlabelled substrate plus 20 μmol of [1-^{14}C]acetate. $G_{exp.}$ is the ratio of the specific radioactivity of the glucose formed to that of the substrate [1-^{14}C]acetate; V_A is the rate of acetate oxidation; V_T is the rate of the tricarboxylic acid cycle. V_G, is the rate of glucose formation, is set equal to 1. $G_{theoret.}$ is calculated from: $G = 2V_A/(V_G + 2V_T + 2V_N)$ (eqn. A5) when lactate is the substrate, and from $G = V_A/(V_G + V_T + V_N)$ (eqn. A6) when lactate is the substrate.

Expt. no.	Unlabelled substrate	Sp. radioactivity of acetate (c.p.m./μmol)	Sp. radioactivity of glucose (c.p.m./μmol)	$G_{exp.}$	V_T	V_A	V_N	$G_{theoret.}$
3	L-Lactate	670000	353000	0.53	2.08	1.39	0.12	0.52
	L-Malate	670000	246000	0.37	1.53	0.98	0.07	0.38
6	L-Lactate	1600000	770000	0.48	2.73	1.64	0.15	0.49
	L-Malate	1650000	630000	0.38	1.60	1.12	0.10	0.41

pools will occur. This will cause production of ^3H in water from L-[2-^{3}H]lactate or L-[2-^{3}H]malate and further dilution of the specific radioactivity of the glucose formed. Although such effects do appear (Table 4), it is impossible to know how much is due to malate exchange or how much to alternative exchange mechanisms (e.g. aldolase, triose phosphate isomerase exchange; Katz & Rognstad, 1966). If the rates of both malate dehydrogenase reactions and of malate exchange flow were infinite, complete isotopic equilibration of cytoplasmic and mitochondrial NAD^{3}H pools would occur. When lactate is the substrate, the rate of mitochondrial NADH turnover is roughly seven times the rate of cytoplasmic NADH turnover, since the rate of the tricarboxylic acid cycle is about 2⅓ times the rate of glucose formation and three NADH molecules are formed in each turn of the tricarboxylic acid cycle. Complete isotopic equilibration of these pools would result in a sevenfold dilution in the specific radioactivity of the glucose formed from L-[2-^{3}H]lactate. Since about a threefold dilution is found, complete isotopic mixing does not occur. One reason for this may be that, although the malate dehydrogenase exchange reactions are active, they are not infinite.

Table 4. *Gluconeogenesis from* L-[2-^{3}H]*lactate and* L-[2-^{3}H]*malate*

For experimental conditions, see Table 1. The specific radioactivity of substrate was set equal to 1.0.

Expt. no.	Substrate	Yield of ^3H in water (% of injected dose)	^3H sp. radioactivity of glucose
1	L-[2-^{3}H]Lactate	80	0.28
	L-[2-^{3}H]Malate	86	0.39
4	L-[2-^{3}H]Lactate	68	0.32
	L-[2-^{3}H]Malate	58	0.31

Again, a corollary of this exchange should be appearance of ^3H in glucose from substrates that produce NAD^{3}H in the mitochondria. Thus, when DL-β-hydroxy[3-^{3}H]butyrate is used as substrate together with lactate or pyruvate, a small amount of ^3H is found in the glucose formed (Table 5). D-β-Hydroxy[3-^{3}H]butyrate forms NAD^{3}H by an enzyme that is thought to be located exclusively in mitochondria (Lehninger, Sudduth & Wise, 1960), whereas L-β-hydroxy[3-^{3}H]butyryl-CoA would form NAD^{3}H via the mitochondrial enzymes that oxidize fatty acids. It is noteworthy that most of the ^3H from DL-β-hydroxy[3-^{3}H]butyrate found in glucose is on C-4 when either lactate or pyruvate is the substrate. This again would be consistent with rapid malate exchange masking any isotopic difference in the net pathways from lactate and pyruvate. However, the interpretation of the results with DL-β-hydroxy[3-^{3}H]butyrate must be considered as very tentative. Other pathways may operate that result in an exchange of reductive hydrogen atoms between the mitochondria and the cytosol, or perhaps some cytoplasmic enzyme reacts with the D- or L-isomer.

Haslam & Krebs (1968) have found that ATP and other high-energy compounds stimulated the entry of malate into isolated liver mitochondria. Our results in the present paper suggest rapid flow of malate through the mitochondrial membrane. Since a high ATP concentration is required for active gluconeogenesis, our results with intact cells do not conflict with their studies on isolated mitochondria. Preliminary experiments with low concentrations of dinitrophenol, which depress but do not abolish gluconeogenesis, indicate no apparent decrease in the rate of intercompartmental exchange.

If one accepts the hypothesis (Lardy *et al.* 1965; Krebs *et al.* 1967) that glucose formation from either pyruvate or L-lactate involves the mito-

Table 5. *Tritium incorporation into glucose from* DL-β-*hydroxy*[3-^{3}H]*butyrate*

For experimental conditions, see Table 1. About 1×10^6 c.p.m. of ^{14}C and 30×10^6 c.p.m. of ^3H were used in each incubation. The $^3H/^{14}C$ ratio of the substrate mixture was set equal to 1.0.

Expt. no.	Substrates (μmol added)	Yield of ^3H in water (% of injected dose)	$^3H/^{14}C$ ratio of glucose	Distribution of ^3H in glucose (%)	
				C-4	C-6
8	L-[2-^{14}C]Lactate (20) + DL-β-Hydroxy[3-^{3}H]butyrate (20)	n.d.	0.030	72	28
	[2-^{14}C]Pyruvate (20) + DL-β-hydroxy[3-^{3}H]butyrate (20)	n.d.	0.024	72	25
2	L-[U-^{14}C]Lactate (20) + DL-β-hydroxy[3-^{3}H]butyrate (20)	28	0.060		
	[U-^{14}C]Pyruvate (20) + DL-β-hydroxy[3-^{3}H]butyrate (20)	29	0.047		
	L-[U-^{14}C]Malate (20) + DL-β-hydroxy[3-^{3}H]butyrate (20)	31	0.041		

chondrial pyruvate carboxylase reaction, it seems likely that malate should flow out of the mitochondria when pyruvate is the substrate, whereas oxaloacetate, or a compound on this oxidation level, should be transferred when lactate is the substrate.

From the difference in the $NAD^+/NADH$ concentration ratios in the cytosol and mitochondria of liver, Krebs (1967) has shown that equilibration of both malate and oxaloacetate between the cytosol and the mitochondria (or at least the mitochondrial space containing malate dehydrogenase) does not occur. Our results showing rapid malate exchange suggest that no control mechanism on malate outflow operates and that malate equilibration between compartments is rapidly established.

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APPENDIX

(*A*) *Specific radioactivity of glucose formed from* L-[U-^{14}C]*lactate*

All models assume metabolic and isotopic steady state. (In some experiments there is a considerable decrease in the rate of gluconeogenesis as the substrate becomes depleted. The approach used is still valid, but in these cases the rates must be interpreted as average flow of carbon during the incubation period.) Scheme 1 in the main paper shows the model used to represent the metabolism of L-[U-^{14}C]lactate by the kidney cortex. Some simplifying approximations have been used: (1) Mitochondrial oxaloacetate, malate and fumarate are assumed to be in complete isotopic equilibrium. (2) The carbon dioxide fixed in the pyruvate carboxylase reaction is assumed to have the same specific radioactivity as the carboxyl carbon of the mitochondrial dicarboxylic acids; this is a good approximation if V_B is small. (3) Recycling of phosphoenolpyruvate back to pyruvate is neglected. (4) The small amount of glucose formed from endogenous sources, V_N, is arbitrarily assumed to be from endogenous glutamate; this is a small inflow (about 5% of V_T).

The specific radioactivity of each carbon atom of L-[U-^{14}C]lactate is set equal to 1. Equations are written below that set inflow of radioactivity into any carbon atom equal to outflow of radioactivity from that carbon atom, assuming an isotopic steady state.

Acetyl-CoA:

$$V_B(1) = aV_T$$

C-4 and C-5 of α-*oxo*glutarate:

$$V_T a = b(V_T + V_N)$$

C-2 and C-3 of α-*oxo*glutarate:

$$V_T m_2 = m(V_T + V_N)$$

C-1 and C-4 of dicarboxylic acids:

$$V_G\left(\frac{1+m_1}{2}\right) + (V_T + V_N)\left(\frac{b+n}{2}\right) = m_1(V_G + V_T + V_N)$$

C-2 and C-3 of dicarboxylic acids

$$V_G(1) + (V_T + V_N)\left(\frac{b+n}{2}\right) = m_2(V_G + V_T + V_N)$$

Solving these equations yields:

$$m_2 = \frac{2V_G + V_B}{2V_G + V_T + 2V_N}$$

and

$$m_1 = \frac{V_G + V_B + V_T m_2}{V_G + 2V_T + 2V_N}$$

The molar specific radioactivity of the glucose formed will be $4m_2 + 2m_1$. Dividing this by 6 gives the ratio (R) of the expected molar glucose specific

activity to that of glucose formed from two C_3 molecules of undiluted specific radioactivity:

$$R = (4m_2 + 2m_1)/6 = (2m_2 + m_1)/3$$

From the values of m_2 and m_1 above:

$$R = \frac{(2V_G + V_B)(2V_G + 5V_T + 4V_N) + (V_G + V_B)(2V_G + V_T + 2V_N)}{3(2V_G + V_T + 2V_N)(V_G + 2V_T + 2V_N)} \quad \text{(A1)}$$

(B) Specific radioactivity of glucose formed from L-[U-^{14}C]*malate*

The model used is shown in Scheme 2. The specific radioactivity of each carbon atom of extramitochondrial malate is denoted by x, and of that of intramitochondrial malate by m. The molar specific radioactivity of the glucose formed will be $6x$. The predicted dilution in specific radioactivity, R, will be $6x/6$ or x. The specific radioactivity of each carbon atom of L-[U-^{14}C]malate is set equal to 1. As above, inflow of radioactivity is equated to outflow, in the isotopic steady state.

Acetyl-CoA:

$$V_B x = aV_T$$

C-4 and C-5 of α-oxoglutarate:

$$aV_T = b(V_T + V_N)$$

C-2 and C-3 of α-oxoglutarate:

$$mV_T = n(V_T + V_N)$$

Intramitochondrial malate:

$$V_E x + (V_T + V_N)\left(\frac{b+n}{2}\right) = m(V_T + V_E + V_N)$$

Extramitochondrial malate:

$$(V_G + V_B)(1) + (V_E + V_N)m = x(V_G + V_B + V_E + V_N)$$

Solving these equations yields:

$$x = R = \frac{(V_G + V_B)(V_T + 2V_E + 2V_N)}{(V_G + V_B + V_E + V_N)(V_T + 2V_E + 2V_N) - (V_E + V_N)(2V_E + V_B)} \quad \text{(A2)}$$

Rearranging to solve for V_E gives:

$$V_E = \frac{(V_T + 2V_N)(V_G + V_B) - R[(V_G + V_B + V_N)(V_T + 2V_N) - V_N V_B]}{R(2V_G + V_B + 2V_N + V_T) - 2(V_G + V_B)} \quad \text{(A3)}$$

If $V_E = \infty$, eqn. (A2) above reduces to:

$$R = \frac{2(V_G + V_B)}{2V_G + V_B + V_T + 2V_N} \quad \text{(A4)}$$

This gives the value of the dilution in glucose specific radioactivity expected if extramitochondrial and intramitochondrial malate are in complete isotopic equilibrium.

(C) Specific radioactivity of glucose formed from [1-^{14}C]*acetate plus either* L-*lactate or* L-*malate*

Schemes 1 and 2 show the models used when L-lactate or L-malate respectively is the unlabelled substrate [however, in this case the specific radioactivities (m_2) of the interior carbon atoms of the dicarboxylic acids and that of C-2 of acetyl-CoA will be zero]. The same approximations as in the previous sections are used. The molar specific radioactivity, G, of glucose formed will be $2m_1$. This is also the ratio of the glucose specific radioactivity to that of [1-^{14}C]acetate, since the latter is set equal to 1. Equations are written for isotopic steady state when lactate is the unlabelled substrate (Scheme 1).

Acetyl-CoA:

$$V_A(1) = aV_T$$

C-5 of α-oxoglutarate:

$$aV_T = b(V_T + V_N)$$

C-1 and C-4 of dicarboxylic acids:

$$(V_G m_1)/2 + (V_T + V_N)b/2 = m_1(V_G + V_T + V_N)$$

Solving these equations yields:

$$m_1 = \frac{V_A}{V_G + 2V_T + 2V_N}$$

$$G = 2m_1 = \frac{2V_A}{V_G + 2V_T + 2V_N} \quad \text{(A5)}$$

When malate is the unlabelled substrate (Scheme 2), we consider only the case where extramitochondrial and intramitochondrial malate are assumed in isotopic equilibrium ($x = m$).

Acetyl-CoA:

$$V_A(1) = aV_T$$

C-5 of α-oxoglutarate:

$$aV_T = b(V_T + V_N)$$

C-1 and C-4 of dicarboxylic acids:

$$(V_T + V_N)b/2 = m(V_G + V_T + V_N)$$

Solving these equations yields:

$$m = \frac{V_A}{2(V_G + V_T + V_N)}$$

$$G = 2m = \frac{V_A}{V_G + V_T + V_N} \quad \text{(A6)}$$

4. Akira Ohara, Katsumi Endo, Shizuko Matsumoto and Masashichi Yoshioka: Studies on the Inhibitors of Thiol-enzymes V. Influence of Dibasic acids on the Activity of Papain.

E. J. Morgan and Friedmann have been shown that maleic acid and SH-compounds interact with the formation of stable addition compounds[1]. They have applied this reaction to the study of some enzyme reactions which are held to be induced by SH-compounds[2].

The combination of α,β-unsaturated ketone with the compounds having SH radical has been already noticed[3]. The inhibiting effects of benzalacetophenone or furfralacetone on papain, was observed by Watanabe et. al[4]. In the previous paper one of the authors described the relation between activity of the thiol enzyme and influence of the several unsaturated compounds[5].

The present research has been planed to clearify relation between activity of the thiol enzyme and influence of the dibasic acids, which have four similar carbon number.

Experiment and Method

1. Papain: Papain used in this experiment was purified by the same method descrived in the previous paper[6]. Analytical data for the preparation are as follows; protein N: 15.17%
2. Substrate: Gelatin (N: 15.33%) was used as substrate.
3. Dibasic acids used in this experiment were shown in Table I.

Table 1. Dibasic acids.

Name	Structure	Name	Structure
succinic acid	CH_2-COOH CH_2-COOH	maleic acid	CH-COOH ‖ CH-COOH
malic acid	HO-CH-COOH CH_2-COOH	dihydroxymaleic acid	HO-C-COOH ‖ HO-C-COOH
tartaric acid	HO-CH-COOH HO-CH-COOH	fumaric acid	HOOC-C-H ‖ H-C-COOH
dihydroxytartaric acid	OH HO-C-COOH HO-C-COOH OH	dihydroxyfumaric acid	HOOC-C-OH ‖ HO-C-COOH
		oxaloacetic acid	CH_2-COOH CO-COOH

4. Determination of activity of enzyme: The Formol titration method[7] described in the previous paper[6] was applied.

Table 2. Influence of Saturated Dibasic Acids on the Activity of Papain.

(M)	Succinic acid 30 –COOH μg equivalent*	Succinic acid 30 activity %	Succinic acid 60 –COOH μg equivalent*	Succinic acid 60 activity %
0	13.4	100.0	17.3	100.0
10^{-5}	15.7	117.2	18.4	106.4
10^{-4}	15.2	113.4	16.5	95.4
10^{-3}	14.4	107.5	16.5	95.4
10^{-2}	11.4	85.1	13.2	76.3

(M)	tartaric acid 30 –COOH μg equivalent*	tartaric acid 30 activity %	tartaric acid 60 –COOH μg equivalent*	tartaric acid 60 activity %
0	13.4	100.0	17.3	100.0
10^{-5}	13.9	130.7	14.6	84.4
10^{-4}	13.4	100.0	14.4	83.2
10^{-3}	11.0	82.1	12.4	71.7
10^{-2}	6.9	51.5	9.2	53.2

(M)	malic acid 30 –COOH μg equivalent*	malic acid 30 activity %	malic acid 60 –COOH μg equivalent*	malic acid 60 activity %
0	13.4	100.0	17.3	100.0
10^{-5}	13.5	100.7	21.4	123.7
10^{-4}	14.1	105.2	22.4	129.5
10^{-3}	14.3	106.7	22.5	130.1
10^{-2}	15.2	113.4	23.0	132.9
10^{-1}	15.9	118.7	27.8	160.7

(M)	dihydroxytartaric acid 30 –COOH μg equivalent*	dihydroxytartaric acid 30 activity %	dihydroxytartaric acid 60 –COOH μg equivalent*	dihydroxytartaric acid 60 activity %
0	13.4	100.0	17.3	100.0
10^{-5}	11.7	87.3	12.3	71.1
10^{-4}	11.4	85.1	12.7	73.1
10^{-3}	9.8	73.1	10.4	60.1
10^{-2}	6.2	46.3	6.8	39.2
10^{-1}	4.3	32.1	5.4	31.2

* per papain N 1mg

Results and Discussion

The effects of dibasic acids upon activity of papain are shown in Table I and Table II.

Succinic acid in the hight concentration inhibited the activity of papain and little inhibited with the decrease of concentration.

Malic acid which was gained by substituting one hydrogen atom of succinic acid with hydroxyl group, increased the activating effect with increase of concentration.

Tartaric acid whichsubstituted one hydrogen atom of malic acid by hydroxyl group, increases the inhibition with the increase of concentration like as succinic acid.

Dihydroxytartaric acid which substituted two hydrogen atoms of tartaric acid with two hydroxyl groups, inhibited the papain activity.

From the above, it was obseved that when substituted hydrogen atom of succinic acid with hydroxyl group, one hydroxyl substitution product increased papain activity but with the increase of hydroxyl groups the inhibition of papain increased in more than two substituents.

Meleic acid having unsaturated bond against succinic acid, showed the strong inhibition upon papain activity. This result is in accord with the report of E. J. Morgan and

Table 3. Influence of Unsaturated Dibasic Acids on the Activity of Papain.

(M)	maleic acid			
	30		60	
	-COOH μg equivalent*	activity %	-COOH μg equivalent*	activity %
0	13.4	100.0	17.4	100.0
10^{-5}	12.3	91.8	14.8	85.1
10^{-4}	10.2	76.1	14.4	82.8
10^{-3}	8.6	64.2	13.8	79.3
10^{-2}	4.8	35.8	8.1	46.6
10^{-1}	3.6	27.6	5.4	31.0

(M)	fumaric acid			
	30		60	
	-COOH μg equivalent*	activity %	-COOH μg equivalent*	activity %
0	13.4	100.0	17.4	100.0
10^{-5}	12.6	94.0	15.2	87.4
10^{-4}	15.2	113.4	20.8	119.5
10^{-3}	15.8	117.9	22.5	129.3
10^{-2}	21.4	159.7	25.2	144.8

* per papain N 1mg

(M)	dihydroxymaleic acid			
	30		60	
	-COOH μg equivalent*	activity %	-COOH μg equivalent*	activity %
0	13.4	100.0	17.3	100.0
10^{-5}	15.0	111.1	18.3	105.7
10^{-4}	11.7	87.3	13.9	80.4
10^{-3}	9.1	67.9	10.3	58.4

(M)	dihydroxyfumaric acid			
	30		60	
	-COOH μg equivalent*	activity %	-COOH μg equivalent*	activity %
0	13.4	100.0	17.3	100.0
10^{-5}	13.8	103.0	19.4	112.1
10^{-4}	14.1	105.2	20.4	117.9
10^{-3}	15.1	112.7	21.3	123.1

(M)	oxaloacetic acid			
	30		60	
	-COOH μg equivalent*	activity %	-COOH μg equivalent*	activity %
0	13.4	100.0	17.3	100.0
10^{-5}	14.2	106.0	20.3	117.3
10^{-4}	18.1	135.1	26.5	153.1
10^{-3}	18.4	137.3	27.1	156.6
10^{-2}	19.1	142.5	27.8	160.6
10^{-1}	26.4	197.0	29.7	171.6

E. Friedmann that maleic acid and -SH compounds interact with the formation of stable addition compounds[1]. That is the inhibition increased with the increase of concentration of maleic acid.

Dihydroxymaleic acid substituting two hydrogen atoms of maleic acid with two hydroxyl groups inhibited stronger than maleic acid. Namely, products substituting hydrogen atom with hydroxyl group, inhibited upon papain activity.

Fumaric acid and dihydroxyfumaric acid, which is *trans* form of maleic acid and dihydroxymaleic acid, showed the activationforpapain and the activation of dihydroxyfumaric acid is stronger than fumaric acid. It is supposed that this is th effect of hydroxyl group.

The order which showed activation among effects of each kinds of dibasic acids upon papain is following:

fumaric acid>malic acid>dihydroxyfumaric acid

Succinic acid showed little influence and the order which showed the inhibition dihydroxytartaric acid>dihydroxymaleic acid>tartaric acid>maleic acid.

From the above, it was observed that unsaturated bond of dibasic acids inhibited for papain activity. It was supporsed that this combinated -SH group of papain in form addition compounds. On substituting hydrogen atom in morecural structure with hydroxyl group, one hydroxyl group activated but more than two inhibited, with the increase of hydroxyl group number inhibited stronger. Fumaric acid, which is *trans* form activated but maleic acid, which is *cis* form inhibited upon papain activity.

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RESEARCH ON THE PHOSPHATASE OF THE PROSTATE.

I.- STUDY ON THE MECHANISM OF ACTION OF CITRIC ACID.

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The phosphatase of the prostate was characterized for the first time by KUTSCHER and WOLBERGS (1). From 1935 to 1941, in a series of studies, KUTSCHER and his coworkers specified the principal properties of this enzyme (2-5) (optimal pH of action (3), inactivation by certain organic solvents (3), inhibition of concurrent character by the fluor ions (5)) and tried to obtain it in highly purified form.

Since then a great number of researchers have enriched our knowledge of the chemistry and enzymology of prostatic phosphatase. In this work however there exist divergences of opinion on certain points. One of these points, as already pointed out by LUNDQUIST (6) is the optimal pH of this enzyme. KUTSCHER and WÖRNER (2) found an optimum pH range extending from 5.2 to 6.2 with betaglycerophosphate as substrate and of pH 4.0 to 5.4 with phenylphosphate.

LUNDQUIST (6) fixes this optimal pH at 6.0 for the two substrates; the curves published by ABUL FADL and KING (7) indicate an optimum at pH 5.2 - 5.5. The curves presented by all these authors have very different forms.

Certain organic anions seem to enter into play in apparently fairly complex manner: LUNDQUIST (6) points out that the citrate ions accelerate the hydrolysis of the beta-glycerophosphate (from 10 to 30 percent and sometimes even more); on the other hand they exert but an insignificant action on the hydrolysis of phenyl-phosphate.

The same author has furthermore observed that the citrate ions (at pH 6.0) neutralize the inhibiting action of small doses of fluoride on the same enzyme and diminish the action of larger doses. He also pointed out the inhibiting action of oxalate and maleinate ions. The action of the oxalate ions is not influenced by the presence of the citrates. The activating

action of the citrate ions on certain acid phosphatases has already been envisioned by different authors (8, 9, 10, 11, 12, 13). Oxalate ions are known inhibitors of the same acid phosphatases (14, 15).

ABUL-FADL and KING (16, 7) observed that the tartrate L (+) ions (the natural dextrorotatory isomer) are energetic inhibitors of prostatic phosphatase and other animal acid phosphatases (liver, bile). These authors pointed out the reversible character of this inhibition.

In our research on the effectors of acid phosphatases (17, 18, 19, 20) we frequenctly resorted to preparations of prostatic phosphatase; we noticed the properties which relate them to the group of acid phosphatases; in addition to the optimum pH, identical mechanism of inhibition by the fluor ions and some mineral polyacids (molybdenum, tungsten, phospho-molybdenum, phospho-tungsten and vanadium). On the other hand we pointed out the characters which differentiate it from the other phosphatases of type II, particularly those of vegetal origin; greater sensitivity to the action of certain reagnets of amine groups (ketene, nitrous acid)(18), wider field of action (action on phosphoric esters of amino-alcohols, hydrolized to a minimal extent by the other acid phosphatases)(21).

It is during our research on the inhibition of acid phosphatases by the fluor ions, while studying the influence of these ions on prostatic phosphatase in function of the pH, that we were led to approach the question of the optimum pH of this enzyme and the role of the citrate ions.

We deemed it interesting to undertake further research on the mechanism of action of the organic anions; we can in fact envision that the study of these phenomena carried out on purified preparations of prostatic phosphate could help us elucidate the complex problems posed by the action of the other effectors of prostatic phosphatase. These results were apt to be generalized to other acid phosphatases. Moreover the prostatic phosphatase represents the most active preparation of acid pohsphatase known to date.

In the first part of this work we shall present the results obtained on the activating action of some organic anions and more particularly on that (the

most important) of the citrate ions.

EXPERIMENTAL PART

a) Enzyme preparations:

1) Prostatic phosphatase. W used preparations obtained by ammonium sulfate fractionation of human prostate autolysates obtained by the following method:

Fresh glands are ground in a Turmix mixer with 10 times their weight of a 0.9% ClNa solution; this is autolyzed for 48 hours at laboratory temperature, (after addition of a few drops of toluene). After centrifuging and filtering on paper, the clear liquid is fractionated, at 0°, by the ammonium sulfate. The fraction obtained, between 60 and 80% of saturation in ammonium sulfate, is washed by a solution of ammonium sulfate at 80% saturation, redissolved in 100 cc of bi-distilled water, then the liquid is subjected to a dialysis of long duration in cellophane bags, at 0°, against the bi-distilled water until complete elimination of ammonium sulfate (negative reaction of the ammonium ions with the NESSLER reagent). The enzyme solution is then diluted so that 1.0 cc, in the conditions described further down, causes a hydrolysis of beta-glycerophosphate of 10 to 20% in 1 hour. These preparations (after dilution) yield 50 to 200 gamma of dry residue per cc depending on the case. They can be stored in the refrigerator under toluene without appeciable loss of activity for several months. The fractions precipitated between 40 and 60% of saturation in ammonium sulfate entrain a certain portion of their phosphatase activity, but it is the insoluble fraction between 60 and 80% which has the greatest activity by far.

2) Other acid phosphatases. When we incidently compared the behavior of prostate phosphatase with that of other acid phosphatases, we used the following phosphatase preparations: phosphatase of White Mustard seed and of Wheat Bran, purified according to our usual method, previously described (17), as representatives of vegetal phosphatases of type II; takadiastase of PARKE, DAVIS and Co. and a macerated mycelium of Aspergillus Niger for phosphatases of type III (opt. pH 4.0), and finally morning urines of man and woman subjected

to 24 hour dialysis in the refrigerator, as a source of animal phosphatase of type II.

b) Determination of enzymatic activity:

In most tests we used disodium beta-glycerophosphate as a substrate. The solutions of this substrate were brought to the pH of the tests by addition of 2 N acetic acid. The concentration of substrate in the medium was 0.016 M. The medium was buffered with 5.0 cc of an acetic acid/sodium acetate buffer (concentration of acetate in the medium - 0.10 M). For a large number of tests in presence of certain effectors this pH was 4.6, experience having taught us that at this pH the activity of the enzyme was still considerable and the action of the effector in question was more manifest than at pH 5.5, which facilitated the study of this phenomenon. The quantity of enzyme solution added was 1.0 cc, as previously indicated, the total volume of each test was 50 cc, the duration of hydrolysis was 1 hour at 37°, the tests having been brought to this temperature before the addition of the enzyme. In the cases where certain of these factors were made to vary, the new conditions adopted for these cases will be specified in the corresponding Tables and Graphs.

c) Curves of activity in function of the pH:

The substrate (beta-glycerophosphate) was brought to the desired pH's by means of 2 N acetic acid. As buffers we used a range of 2 M acetic acid/sodium acetate buffers. For the pH range from 5.5 to 6.5 we used the buffering power of the substrate. We took account of the variations in ionic forces and, following LUNDQUISTS's (6) recommendations, we maintained the ionic force constant in all the tests of each series by adding to the solutions of acetic acid which served for the preparation of the buffers, an equimolar quantity of ClNa.

The pH of the tests was controlled with the glass electrode. For the curves in function of the pH, in presence of citrate, we used disodium citrate/hydrochloric acid and disodium citrate/soda buffers according to SÖRENSEN covering the range of pH from 2.5 to 6.5 at variable quantities to maintain the concentration in citrate constant along the entire pH range under study.

Precautions were taken to also maintain the ionic force constant.

The other conditions were those set forth under (b).

d) Study of the action of effectors:

The solutions of pure substances (organic acids, metal salts or other effectors) brought to the pH of the tests by means of 2 N acetic acid or soda were added in the medium containing the substrate and the buffer, before the addition of the enzyme in quantities suitable to obtain the desired effector concentration. In certain cases there was prior incubation of the enzyme with the effector. In these cases, or when particular precautions were necessary, these details are specified in the text.

On several occasions we had to dose small quantities of citric acid (in raw prostate autolysates, in phosphatase preparations dialyzed in presence of citric acid); in these cases we used the WEIL-MALHERBE and BONE (22) micromethod with slight modifications in the handling details. The elimination of protein matter was done with the FOLIN and WU reagent.

RESULTS

Action of the citrate ions in function of the pH.

Fig. 1 shows the influence of the pH on the activation of prostate phosphatase by the citrate ions. This activation, of small extent in the optimum pH region of the enzyme (pH 5.6) and at greater pH values, becomes more important as the pH decreases. The prostate phosphatase exhibits a considerable activity in the range of pH 3.6 - 6.0 while in the absence of citrate the activity quickly drops from a pH more acid than 5.0.

The curve obtained in presence of citrate is flattened in the range of pH 3.5. 6.0, the optimum (pH 5.54) is not very prominent (the percentage of hydrolysis of the substrate is 13.8 for a pH 3.6 to 15.1 for pH 5.54). This curve resembles that obtained with phenylphosphate, without addition of citrate (Fig. 2), substrate for which prostate phosphatase has a greater affinity than for beta-glycerophosphate and on the hydrolysis of which the citrate ions have little action (see also Table IV, further down).

Moreover, the hydrolysis curve of beta-glycerophosphate by prostate phospha-

tase in function of the pH, in presence of citrate ions, recalls the curve published by KUTSCHER and WÖRNER (3) representing the stability of prostate phosphatase in function of the pH.

The rate of activation of the enzyme by the citrate at a given pH (especially for pH values below pH 5.0) varies slightly from one enzyme preparation to the other. This is probably in relation with the degree of denaturation of the enzyme. We shall return to this point later on.

Table I indicates the percentage of activation (at pH 4.6) by variable citrate concentrations which we observed with most of our freshly prepared purified preprations following our operating method, i.e. with enzymes subjected to the treatment indicated above (experimental part (a)).

It can be seen that the citrate ions manifest their action beginning with a small concentration (10^{-5} M); at a concentration of 10^{-4} M the action is already considerable; the optimal concentration is approximately 10^{-2} M; at higher concentrations (10^{-1} M) the activation decreases.

On preparations kept too long in the refrigerator (more than one year) and which have lost a part of their activity through aging, the action of citrate ions seems more pronounced. On raw prostate autolysates, the action of citrate is less marked than on purified preparations. This is explained by the fact that these raw autolysates contain small quantities of citric acid, a natural component of the prostatic secretion.

In a dilute prostate autolysate we found a concentration of 8.9×10^{-5} M citric acid.

Fig. 3 represents the hydrolysis of beta-glycerophosphate in function of the pH by such a fresh prostate autolysate with and without addition of citrate.

The curve obtained with the autolysate without citrate occupies an intermediate position between the purified preparation without addition of citrate (Fig. 1, curve 1) and the 2 almost identical curves obtained with the purified preparation or autolysate in presence of a concentration of 1×10^{-2} M citrate (Fig. 1 and 2, curves II).

Action of various organic compounds on prostatic phosphatase:

We have sought if this activating action was specific of the citrate ions and what could be the chemical function of this body responsible for this activation.

To this end, we studied the action of a great number of compounds on the hydrolysis of beta-glycérophosphate by the prostate phosphatase: mono- and di-carboxyl organic acids, alcohol acids, acids possessing other functions (thiol, amine, ketone), citric acid derivatives (dimethyl ester of citric acid), polyols, oses,

Table II shows that only carboxyl compounds possessing in addition an alcohol thiol or ketone function at alpha, are capable of causing the activation of the enzyme. Thus lactic acid and alpha-oxybutyric acid have an activating action, but beta-oxypropionic acid and beta-oxybutyric acid exert no activation; dimethyl ester of citric acid, which differs from citric acid only by the fact that two of the carboxyls are blocked by esterification, acts like citric acid. A thiol or ketone function (the latter reacting probably under its enolic form) at alpha imparts to the product the same power of activation on the prostate phosphatase. The oses and polyols studied exerted no action.

The alpha-amine acids cause a slight inhibition and this action is due to the amine function, as demonstrated by tests with monoethylamine and hydrazine. Certain dicarboxyl acids possessing an even number of carbon atoms (oxalic, succinic, maleic) exert a certain inhibition.

We shall study the action of L-tartric acid further on.

The citrate ions however possess thepower of activation on phosphatase to a much high r degree than all the other alpha-alcohol acids etc.; free carboxyls are necessary, as shown by the experiments whose results appear in Table III, where the action of some alpha-alcohol acids on the enzyme has been studied in function of the concentration of the effector.

In fact, comparing Tables I and III, we can observe that glycolic and lactic acids have little action at low concentrations, and the same holds true

for the dimethyl ester of citric acid, with respect to citric acid.

Kinetics of activation by citrate ions:

We studied in greater detail the mechanism of this activation by citrate ions. In Fig. 4, according to the method of presentation of LINEWEAVER and BURK (23), are represented the data obtained in function of the concentration in substrate (beta-glycerophosphate) with and without addition of citrate.

According to these experiments, the citrate ions increase the affinity of the enzyme for the substrate. The affinity of prostate phosphatase for beta-glycerophosphate at pH 4.6 calculated according to this diagram is 47.5 in the absence of citrate and 145.5 in presence of a concentration of 1×10^{-2} M citrate. With strong concentrations of substrate (above 0.02 M) the speed of hydrolysis in presence of citrate decreases a little. This fact seems to indicate a competition between the substrate and the effector for the same active groups of the enzyme.

The tests performed in function of the duration of hydrolysis and in function of the enzyme concentration (Fig. 5) show that a competition also exists between the citrate ions and the phosphate ions released. The purified enzyme does not appear to be affected by small quantities of phosphate. On the other hand these same phosphate concentrations decrease the activation produced by the citrate. The strongest activation at low rates of hydrolysis of the substrate (125% activation at 4.3% hydrolysis of the substrate in the control test) decreases as the hydrolysis of the substrate progresses (20% activation only when the hydrolysis is 47% in the control test). The citric activation depends moreover on the enzyme concentration in the hydrolysis medium.

Action of citrate on the hydrolysis of different substrates:

Table IV shows that the citrate ions increase the speed of hydrolysis of all the substrates we subjected to the action of prostatic phosphatase, but the rate of activation varies according to the substrate (for the same molecular concentration in substrate and the same percentage of hydrolysis). This rate of activation is in relation with the affinity of the enzyme for the

substrate in question: the activation is all the more pronounced as the affinity K_M for the substrate is smaller.

Action of citrate ions on other acid phosphatases:

Prostate phosphatase, although more sensitive to the action of the citrate ions, is not the only phosphatase apt to be activated by this effector. Outside of phosphatases of Type III (taka-diastase, Aspergillus niger) whose activation by citrates has already been studied by BAMANN and SALZER (9, 10) and COURTOIS (11) the vegetal acid phosphatases exhibit this same property, though to a much smaller extent.

In this Table V are compared the activities of all these enzymes in presence of citrate.

In order to explain the nature of the zction of citrate ions on the affinity of prostate phosphatase for its substrates and on the speed of hydrolysis of these substrates, we have envisioned two hypotheses:

- The citrate ion would be either a "true activator" of the enzyme or an "anti-inhibitor". In the second case, it would act by blocking an inhibitor present in the reaction medium; it would be contributed either by the enzyme preparation itself, or by the reagents (substrate, buffer, etc.).

The use of bi-distilled water prepared in a glass apparatus for the preparation of all the solutions and of pure products "for analysis", and controlled by us also for the presence of certain ions should, in principle, make it possible to eliminate this last eventuality. There remained the hypothesis of a natural inhibitor existing in the enzyme preparations as an impurity and, as such, we envisioned traces of metallic elements so easily complexed by alpha-alcohol acids and present almost regularly in the ashes of prostate phosphatase preparations.

In a purified preparation of prostate phosphatase we were able to discover the following metallic elements by spectrography:

Mg, Ca, Fe (the latter in infinitely small traces).

These metals solidly adsorbed on the proteins and, by this fact not eliminated by dialysis, would hinder the formation of the enzyme/substrate complex

and their elimination (or blocking) by the citrate would increase the affinity of the enzyme for the substrate.

Dialysis in presence of citrate:

In attempting to elucidate this problem: does the citrate act as an activator of phosphatase or as an anti-inhibitor by blocking an inhibitor present in the medium?, we resorted to dialysis tests.

Solutions of prostate phosphatase were mixed in equal volumes with a citric buffer M/10 of pH 4.6 and the mixtures were dialyzed at $+3^{\circ}$ against bi-distilled water, frequently renewed. Elimination of the citrate ions was followed by daily qualitative search and frequent microdosages in the dialysate. Dialysis was continued for two or three more days after the dialysate had ceased to produce the citrate reaction according to the micromethod of WEIL-MALHERBE and BONE (21). Control solutions of phosphatase diluted with the same volume of acetic buffer of pH 4.6 were subjected to dialysis of like duration.

During the dialysis, the preparation remaining in the dialyzer was often controlled from the standpoint of enzymatic activity, with and without addition of citrate. Table VI represents the principal steps of this operation.

The major portion of the citrates is eliminated during the first few days. The elimination then becomes slower and slower. A small quantity persists in the medium and it is eliminated only very slowly. A certain proportion always remains bound to the enzyme preparation, even after 46 days of dialysis when the concentrated dialyzate has not produced the citric acid reaction for several days.

The activity of the enzyme solution dialyzed in presence of citrates decreases as the citrate is eliminated from the medium and the enzyme can be reactivated by addition of a new quantity of citrate.

This would prove that the citrates act not only by eliminating an inhibitor present in the medium. In fact the percentage of activation by a new quantity of citrate depends on the duration of the dialysis, i.e. on the quantity of citrate still present in the liquid subjected to dialysis. Nevertheless the activity of the enzyme dialyzed in presence of citrate never drops back to the

initial value of the phosphatase activity (i.e. before the adition of citrate). A certain activation persists even after a very prolonged dialysis (approximately 44% activation after 40 days of dialysis). It seems logical to attribute this activation to small quantities of citrate (2.85 by 10^{-5} M in the preparation dialyzed for 40 days) which do not pass through the membrane and appear to be bound to the enzyme. It should be mentioned however that a preparation of purified prostate phosphatase, and therefore containing not even traces of citrate, placed in presence of a quantity of citrate twice as great as that found in lengthily dialyzed preparations (added in the hydrolysis medium or incubated with the enzyme for 8 days in the refrigerator) was activated only by 6.5%. It is quite possible that the dialyzed enzyme having undergone a long contact with a very large quantity of citrate, has fixed a small quantity of this ion, which is solidly fixed upon the enzyme and facilitates the bond with the substrate.

Influence of citrates on the inhibition of phosphatase by fluorides:

Table VII shows that the citrate ions can either abolish the inhibition of prostate phosphatase produced by small concentrations of fluor ions (2×10^{-4} or less), or diminish that produced by greater concentrations of this inhibitor. In order for the citrate ions to fully exert this action, they must exist in large excess: the ratio fluoride/citric acid must be equal to 1:50. Finally this action depends on the pH of the medium.

Fig. 6 shows an example of the action of fluor ions on prostate phosphatase in function of the pH, in presence and in absence of citrate ions.

In the absence of citrate, for a given concentration of fluor ions, the inhibition has a stable value for all pH values less than 4.5..

Beyond this pH (4.5), inhibition of the enzyme decreases gradually. On the other hand the citrate ions fully exert their fluorated inhibition only at pH values greater than 4.5. For lower pH values, the action of citrates becomes weaker and weaker and the fluorated inhibition approaches the value obtained in the absence of citrate.

This action is specific for citrate ions and could be attributed to the ex-

istence of several carboxyls in the citric acid molecule, in other alpha-alcohold acids, etc., which activate the phosphatase. A certain number does not exert this action at all on fluorated inhibition and the others do exert it, but to an extent much smaller than that of citric acid. The blocking of the two carboxyls of citric acid (dimethyl ester of citric acid) produces a great decrease of this action (Table VIII).

The citrates do not influence the inhibition of vegetal phosphatases by the fluor ions.

GENERAL DISCUSSION OF RESULTS

We have discussed above, in connection with each Table or Figure, the facts contributed by these experiments. From the body of these results the following facts stand out:

1) Organic acids possessing an alcohol, thiol or ketone function at alpha increase the affinity of human prostate phosphatase (and of certain other acid phosphatases) for its substrates. The existence of severa l carboxyls in the molecule accentuates this property of the alpha-alcohol acids, etc., and citric acid with its 3 carboxyls proves the most effective of all the acids studied. This action is probably, to a large extent, an anti-inhibiting action: elimination of an inhibitor present in trace quantities in even the most purified enzyme preparations which are partly free in the medium (dissociated and apt to be eliminated by dialysis in presence of citrate ions) and partly solidly fixed upon the enzyme or even forming a part of the enzymatic molecule. We cannot yet pronounce ourselves concerning the nature of this inhibitor. The metallic elements (Ca, Mg, Fe) present in very small quantities in the ashes of the enzyme preparations used can probably be the cause of this phenomenon.

2) The tests performed on purified preparations of prostate phosphatase using phenylphosphate as a substrate and the tests with other substrates in presence of citrate ions bring out the fact that this enzyme exhibits an activity in a rather extensive range of pH (pH 3.6 - 6.2); with a very little marked optimum (towards pH 5.54).

This fact is interesting from the standpoint of the physiological action of prostate phosphatase. This phosphatase can be particularly active in the vaginal medium and withstand, without appreciable reduction of its activity, the pH variations of this environment (pH 4.0 - 5.6) as was earlier pointed out by KUTSCHER and WÖRNER (3) in connection with their tests on the thermic inactivation of the enzyme in function of the pH. The presence of important quantities of citrate in the sperm guarantees the stability and the activy of the enzyme in this pH range.

3) The action of the citrate ions on the inhibition of prostate phosphatase by fluor ions deserves being discussed in greater detail. We cannot yet affirm whether a relation does or does not exist between the activating or anti-inhibiting action of the citrate ions and their power to abolish (or decrease, according to the case) the inhibition produced by the fluorides. Yet we had indicated that the citrate ions are the only ones, among the organic anions studied, to possess this power to a high degree.

On the other hand, the existence of several carboxyls in the citric acid molecule seems to be the cause of this state of affairs. The other alpha-alcohol acids, etc. have little action on the fluorated inhibition.

The citrate, as we have already seen, is the only ion among all the anions studied possessing to the highest degree the activating or anti-inhibiting power over phosphatase. It could well be that, along with the inhibitor contained in their midst, the fluor ions form a complex which has a greater inhibiting power than the fluor ions alone. By combining with the inhibitor and eliminating it from the medium, the citrate ions would reduce the inhibition of the enzyme by the fluor ions to its true value: combination enzyme + fluoride, and no longer enzyme + inhibitor + fluoride. The unknown inhibitor would have more affinity for the fluor ions than for the citrate ions, as can be imagined from the fact that a great excess of citrate is always necessary to abolish (or diminish) the inhibition exerted by the fluor ions.

Curve 1 of Fig. 6 represents the dissociation in function of the pH of the enzyme complex + fluoride (or enzyme + inhibitor + fluoride) or that of the

complex fluoride + unknown inhibitor.

An interesting fact is that the pH value at which the inhibition exerted by the fluor ions begins to diminish (without addition of citrate) is pH 4.4, which corresponds to the value found by KUTSCHER and PANY (4) for the isoelectric point of the prostatic enzyme.

This fact would mean that if the curve represents the dissocation of the complex enzyme + fluoride in function of the pH, this complex is more stable on the acid side of the isoelectric point of the enzyme.

The inhibition by the fluor ions has a fixed value for each concentration in fluor ions for all pH values more acid than the isoelectric point of the phosphatase. From this pH the complex begins to dissociate and the inhibition diminishes. The opposite is true for the citrate ions: the combination citrate + unknown inhibitor or citrate + enzyme which protects the pohsphatase from the fluor ions is more stable on the alkaline side of this same pH value (pH 4.4 - 4.5); for more acid pH values this combination issociates and the citrate ions are less and less effective for the protection of the enzyme against the fluor ions. Now, our experiments of dialysis of prostate phosphatase in presence of citrate,outlined above, tend to prove that the natural inhibitor (probably a metallic ion) does not dissociate easily and appears to form a part of the enzymatic molecule. This inhibitor would hinder the combination enzyme - substrate and this would occur especially on the acid side of the optimum pH of the enzyme. The combination of fluor ions with this inhibitor would bar the access of the enzyme to the substrate.

It should be supposed that the citrate ions complex this inhibitor on the surface of the enzyme and, simultaneously and independently, foster the formation of the combination enzyme - substrate. This would explain the activating (or anti-inhibiting) action of the citrate ions and at the same time their effect in the case of fluorated inhibition.

A competition would exist between the citrate ions and the fluor ions, which would be influenced by the pH and the relative concentrations in citrate ions and in fluor ions.

We wish to express our thanks to Prof. P. FLEURY and J. E. COUTOIS for the interest they have shown in this work.

S U M M A R Y

1) The action of many organic anions was studied on the phosphatase of purified human prostate. The acids possessing an alcohol, thiol or ketone function at alpha increase the affinity of this enzyme for its substrates. Citrate ions exhibit this action to a greater degree than the other ions studied.

Activation is more manifest on the acid side of the optimum pH of the phosphatase. The curve of affinity of the enzyme in function of the pH has, in presence of citrate ions, a flattened form in the pH range 3.6 - 6.2 and exhibits a slightly pronounced optimum towards pH 5.5 - 5.6.

2) Citrate ions have in addition the power of abolishing the inhibition of the enzyme by small concentrations of fluor ions and of diminishing the inhibition produced by strong concentrations of the same ion. This action is in function of the pH.

Fig. 1. Action of citrate ions on the hydrolysis of beta-glycerophosphate by the phosphatase of purified prostate in function of the pH.

Concentration of citrate ion in the medium: 1×10^{-2} M:

I. Without addition of citrate

II. With addition of citrate.

Fig. 2. Action of citrate ions on the hydrolysis of phenylphosphate in function of the pH, by the phosphatase of purified prostate.

Concentration of citrate in the medium: 1×10^{-2} M.

Duration of hydrolysis: 30 minutes.

I. Controls, without addition of citrate

II. Tests in presence of citrate.

TABLE I. Action of Citrate Ions on Prostate Phosphatase.

Molecular concentration of citrate ions in the medium	$IXI0^{-5}$	$IXI0^{-4}$	$IXI0^{-3}$	$IXI0^{-2}$	$IXI0^{-1}$
Relative activity	110.7	146.2	177.3	180.0	157.0

ph 4.6. - Test method: see experimental part (b).

The figures represent relative activities; ratio of the activity of the enzyme in presence of the effector to that of the control without effector, this ratio being multiplied by 100.

Fig. 3. Action of citrate ions on the hydrolysis of beta-glycerophosphate, in function of the pH, by a non-purified autolysate of human prostate. Concentration of citrate ions in the medium: 1×10^{-2} M.

I. Controls, without addition of citrate

II. Tests with citrate.

TABLE II. Action of certain organic anions (other than citrates) and some other substances on prostate phosphatase.

Nature of the effector	Relative Activity	Nature of the Effector	Relative Activity
D-malate	180.0	glycolate	160.0
D,L malate	85.2	D-lactate	180.0
D-tartrate	180.0	Beta-ocypropionate	100.0
L(*)tartrate	4.8	alpha-oxybutyrate	175.0
D-mucate	173.5	Betz-oxybutyrate	100.0
D-saccharate	173.8	dimethylic ester of citric acid	180.0
Oxalate	70.0	quinate	172.5
Malonate	100.0	pyruvate	150.0
Succinate	84.8	L-cysteine	112.1
Maleinate	81.8	methylamine	83.4
Fumarate	100.0	hydrazine	75.9
Aconinate	100.0	D-glucose	100.0
Thiomalate	180.0	D-levulose	100.0
L-glutamate	87.5	D-mannitol	100.0
L-aspartate	82.35		

pH 4.6.- Acetic buffer. Concentration of effector in the medium: 1×10^{-2} M. For the calculation of relative activity, see Table I.

TABLE III. Action of some alpha-alcohol acids on prostate phosphatase in function of the concentration of effector.

Molecular concentration of effector in the medium	Acid D-lactic	glycolic	dimethyl-citric (dimethylic ester of citric acid
$1X10^{-2}$	180.0	160.0	180.0
$1X10^{-3}$	107.0	135.4	127.0
$1X10^{-4}$	102.0	114.7	107.0

pH 4.6.- Acetic buffer. Substrate: beta-glycerophosphate.

The figures represent the relative activities calculated as in Table I.

Fig. 4. Action of citrate ions on the affinity K_M of prostate phosphatase for beta-glycerophosphate.

pH 4.6.- I. Control tests (without addition of citrate)

II. Tests made in the presence of citrate (1×10^{-2} M)

In abscissae: $\frac{1}{(S)}$ = inverse of the molecular concentration of substrate in the medium.

In ordinates: $\frac{1}{V}$ = inverse of the speed of enzymatic hydrolysis, expressed in mg of phosphorus, released in the form of orthophosphoric acid per minute.

Fig. 5. Influence of the orthophosphates released during the hydrolysis of beta-glycerophosphate by the prostate phosphatase in presence of citrate ions.

In abscissae: molecular concentration of orthophosphate $(PO_4)'''$ in the medium (phosphates released by hydrolysis either in function of time (I) or in function of the enzyme concentration (II)).

In ordinates: relative activity calculated as in Table I, with respect to control tests without addition of citrate.

pH 4.6. Concentration of citrate in the medium: 1×10^{-2} M.

I. Tests in function of the duration of hydrolysis (constant enzyme concentration).

II. Tests in function of the enzyme concentration (duration of hydrolysis

constant. Quantity of enzyme variable: 0.10, 0.50, 1.0, 2.0 cc of enzymatic preparation.

TABLE IV. Actions of citrate ions on the hydrolysis of various substrates by human prostate phosphatase.

Substrate	Duration of hydrolysis (Minutes)	Relative Activity
Beta-glycerophosphate	60	180.1
Phenylphosphate	30	116.1
Morpholinoethanolphosphate	45	129.6

pH 4.6.- Concentration of effector: 1 c 10^{-2} M. The duration of contact of each substrate with the enzyme has been calculated so as to have an hydrolysis of 10% in the control tests (without effector). The other conditions are mentioned in the experimental part (b).

Relative activities calculated as in Table I.

TABLE V. Action of the citrate ions on various acid phosphatases.

Origin of the enzyme	pH of the test	Concentration of enzyme (%)	Relative activity
Takadiastase (Aspergillus oryzae)	4.0	0.10 g.	201.7
Aspergillus niger	4.0	10 cm^3	145.9
Moutarde blanche	4.6	0.02 g.	110.2
Son de Ble	4.6	0.02 g.	114.3

Duration of hydrolysis: 24 hours for all the tests.

Other operating conditions: see experimental part (b).

Relative activities calculated as in Table I.

TABLE VI. Dialysis of prostate phosphatase in presence of citrate ions.

A. Duration of dialysis (days)

B. Molecular concentration of citrate ions in the enzymatic preparation.

C. Search for citrate ions in the dialysate.

D. Activity

E. Activity in presence of citrate ions 1 x 10^{-2} M in the hydrolysis medium

F. Relative activity.

A	B	C	D	E	F
0	0		1446.6	2598.8	179.6
0	$5x10^{-2}$		2339.0	-	-
18	$6.2x10^{-4}$	+	1931.9	2601.6	134.7
40	$2.85x10^{-5}$	-	1907.0	2584.0	135.4

(1) gamma of mineral phosphorus released in the formof orthophosphoric acid per mg of total nitrogen and per minute in the standard conditions specified previously (experimental part (b)). Hydrolysis of the substrate not exceeding 20%.

(2) Calculated as in Table I.

TABLE VII. Influence of citrate ions on the inhibition of prostate phosphatase by fluor ions.

Moleular concentration of the effector in the medium.

PH	FNa	Citrate			
		(1) 0	$2x10^{-4}$	$1x10^{-3}$	$1x10^{-2}$
3.6	$2x10^{-4}$	28.5	--	--	40.5
	$1x10^{-3}$	4.3	--	--	5.5
4.6	$2x10^{-4}$	36.4	36.4	65.5	92.5
	$1x10^{-3}$	0.0	--	--	34.1
5.6	$2x10^{-4}$	66.7	--	--	98.9
	$5x10^{-4}$	8.2	--	--	93.7
	$1x10^{-3}$	5.9	--	--	86.4

These experiments (like those of Fig. 6) were performed without addition of buffer; the inhibition by the fluor ions (at the concentration of 2×10^{-4} M) being much more important in presence of a great concentration of ions in the medium, the citrate ions possessing the faculty of abolishing the inhibition to the same degree in both cases. The solutions of substrate and citric acid were brought to the desired pH by addition of CH_3COOH 2 N or OHNa 2 N. The other operating conditions are those described in the experimental part (b). The hydrolysis of the substrate did not exceed 15%. The figures repre-

sent the relative activities calculated as in Table I; those in absence of citrate ions (10) with respect to controls containing no citrate; thoseof the other columns with respect to control tests containing citrate.

Fig. 6. Action of citrate ions on the inhibition of prostate phosphatase by fluor ions in function of the pH.

Concentration of effectors: FNa: 2×10^{-4} M

Citrate: 1×10^{-2} M

I. Tests without citrate

II. Tests with addition of citrate.

The relative activities are calculated as in Table I; thoseof curve I with respect to a series of control tests without citrate; those of curve II with respect to control tests containing citrate.

The experiments were performed without addition of buffer, like those of Table VII.

TABLE VIII. Influence of different organic anions on the inhibition of prostate phosphatase by fluor ions.

Nature of the organic anion	Molecular concentration or organic anion	Molecular concentration of fluor ions in the medium	
		2×10^{-4}	1×10^{-3}
Aucun	--	36.4	0.0
Citrate	1×10^{-2}	92.5	34.1
D-malate	1×10^{-3}	41.7	--
D-malate	1×10^{-2}	48.4	--
Dimethyl-citrate	1×10^{-2}	56.0	0.0
Aconitate	1×10^{-2}	36.4	0.0
Lactate	1×10^{-2}	36.4	0.0

pH 4.6.- Without addition of buffer.

Experimental conditions described in the experimental part (b). The figures represent the relative activities calculated as in Table I.

Translated by Carl Demrick Associates, Inc./ARB/db

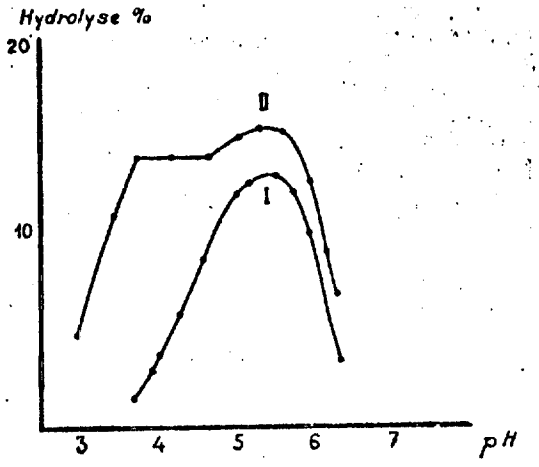


Fig. 1.

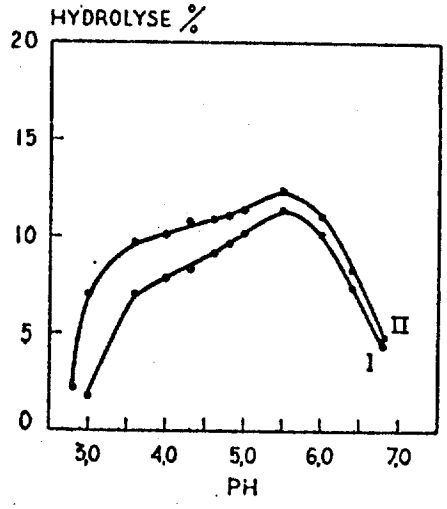
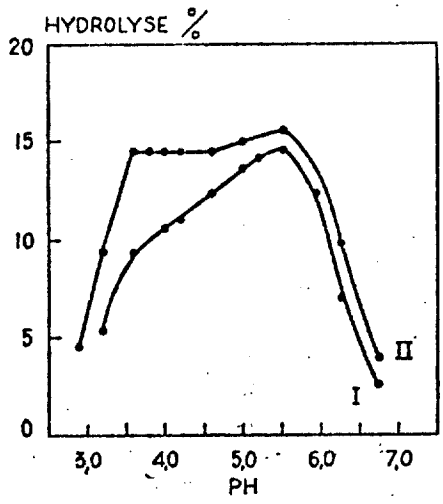


Fig. 2.



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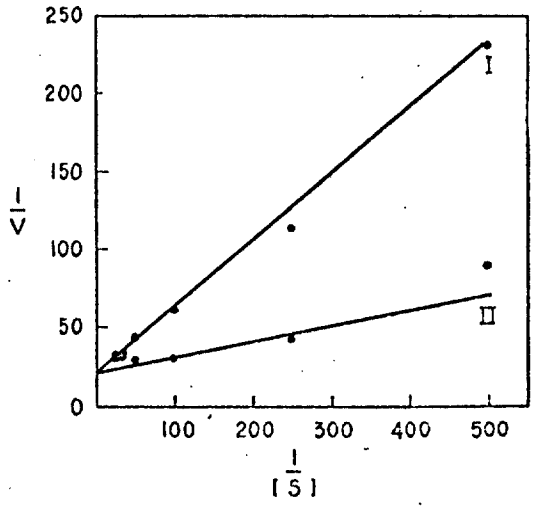

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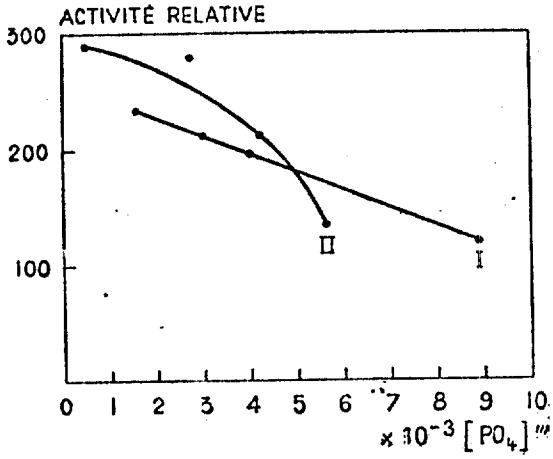

Fig. 5.

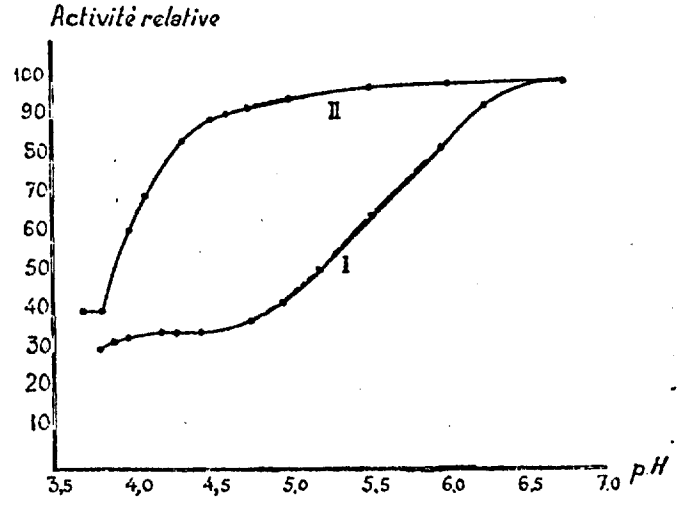

Fig. 6.

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RECHERCHES SUR LA PHOSPHATASE DE LA PROSTATE.
I. — ÉTUDE SUR LE MÉCANISME D'ACTION DE L'ACIDE CITRIQUE.

par C. ANAGNOSTOPOULOS.

(*Mémoire reçu le 10 avril 1953*) (*).

La phosphatase de la prostate fut caractérisée pour la première fois par KUTSCHER et WOLBERGS [1]. De 1935 à 1941, dans une série de travaux, KUTSCHER et ses collaborateurs ont précisé les principales propriétés de cet enzyme [2-5] (pH optimum d'action [3], inactivation par certains solvants organiques [3], inhibition de caractère concurrent par les ions fluor [5]) et se sont efforcés de l'obtenir sous forme hautement purifiée.

Dès lors, un grand nombre de chercheurs ont enrichi nos connaissances sur la chimie et l'enzymologie de la phosphatase prostatique. Il existe pourtant, dans ces travaux, des divergences d'opinion sur certains points. Un de ces points, comme l'a déjà remarqué LUNDQUIST [6] est le pH optimum de cet enzyme. KUTSCHER et WÖRNER [2] ont trouvé une zone pH optimum s'étendant de 5,2 à 6,2 avec le β-glycérophosphate comme substrat et de pH 4,0 à 5,4 avec le phénylphosphate.

LUNDQUIST [6] fixe ce pH optimum à 6,0 pour les deux substrats, les courbes publiées par ABUL FADL et KING [7] indiquent un optimum à pH 5,2 - 5,5. Les courbes présentées par tous ces auteurs ont des formes très différentes.

Certains anions organiques semblent intervenir d'une façon apparemment assez complexe : LUNDQUIST [6] a signalé que les ions citrate accélèrent l'hydrolyse du β-glycérophosphate (de 10 à 30 p. 100 et quelquefois plus), par contre, ils n'exercent qu'une action insignifiante sur l'hydrolyse du phénylphosphate.

Le même auteur a observé de plus que les ions citrate (à pH 6,0) neutralisent l'action inhibitrice des faibles doses de fluorure sur le même enzyme et diminuent celle des doses plus fortes. Il a également signalé l'action inhibitrice des ions oxalate et maléinate. L'action des ions oxalate n'est pas influencée par la présence des citrates. L'action activatrice des ions citrate sur certaines phosphatases acides a déjà été envisagée par différents auteurs [8, 9, 10, 11, 12, 13]. Les ions oxalate sont des inhibiteurs connus des mêmes phosphatases acides [14, 15].

ABUL-FADL et KING [16, 7] ont observé que les ions L(+) tartrate (l'isomère dextrogyre naturel) sont des inhibiteurs énergiques de la

(*) Une partie de ce travail a été présentée à la séance du 14 février 1951.

phosphatase prostatique et d'autres phosphatases acides animales (foie, rate). Ces auteurs ont signalé le caractère réversible de cette inhibition.

Dans nos recherches sur les effecteurs des phosphatases acides [17, 18, 19, 20] nous avons fréquemment eu recours à des préparations de phosphatase prostatique ; nous avons remarqué les propriétés qui la rattachent au groupe des phosphatases acides : outre le pH optimum, mécanisme identique d'inhibition par les ions fluor et quelques polyacides minéraux (molybdique, tungstique, phosphomolybdique, phosphotungstique et vanadique). D'autre part, nous avons signalé les caractères qui la différencient des autres phosphatases du type II particulièrement de celles d'origine végétale : plus grande sensibilité à l'action de certains réactifs des groupements aminés (cétène, acide nitreux) [18], champ d'action plus étendu (action sur les esters phosphoriques des amino-alcools, hydrolysés d'une façon minime par les autres phosphatases acides) [21].

C'est au cours de nos recherches sur l'inhibition des phosphatases acides par les ions fluor, en étudiant l'influence de ces ions sur la phosphatase prostatique en fonction du pH, que nous avons été conduits à aborder la question du pH optimum de cet enzyme et le rôle des ions citrate.

Nous avons estimé qu'il serait intéressant d'entreprendre des recherches plus approfondies sur le mécanisme d'action des anions organiques ; nous pouvons en effet envisager que l'étude de ces phénomènes poursuivie sur des préparations purifiées de phosphate prostatique pourrait nous aider à élucider les problèmes complexes posés par l'action des autres effecteurs de la phosphatase de la prostate. Ces résultats pouvaient être susceptibles d'être généralisés à d'autres phosphatases acides. D'ailleurs la phosphatase de la prostate représente la préparation la plus active de phosphatase acide actuellement connue.

Dans la première partie de ce travail, nous présenterons les résultats obtenus sur l'action activatrice de quelques anions organiques et plus particulièrement sur celle (la plus importante) des ions citrate.

Partie expérimentale.

a) *Préparations enzymatiques :*

1) *Phosphatase prostatique :* Nous avons employé des préparations obtenues par fractionnement au sulfate d'ammonium des autolysats de la prostate humaine obtenus selon le protocole suivant :

Les glandes fraîches sont broyées dans un mélangeur Turmix avec 10 fois leur poids d'une solution de ClNa à 0,9 p. 100 : nous laissons à autolyser pendant 48 heures à la température du laboratoire, (après addition de quelques gouttes de toluène). Après centrifugation et filtration sur papier, le liquide clair est fractionné, à 0°, par le sulfate d'ammonium. La fraction obtenue, entre 60 et 80 p. 100 de saturation en sulfate d'ammonium, est lavée par une solution de sulfate

d'ammonium à 80 p. 100 de saturation, redissoute dans 100 cm³ d'eau bi-distillée, puis le liquide est soumis à une dialyse de longue durée dans des sacs de cellophane, à 0°, contre de l'eau bi-distillée jusqu'à élimination complète de sulfate d'ammonium (réaction négative des ions ammonium avec le réactif de Nessler). La solution enzymatique est alors diluée de façon à ce que 1,0 cm³ dans les conditions décrites plus loin, provoque une hydrolyse de β-glycérophosphate de 10 à 20 p. 100 en 1 heure. Ces préparations (après dilution) fournissent de 50 à 200 γ de résidu sec par cm³ suivant le cas. Elles peuvent être conservées à la glacière sous toluène sans perte appréciable de leur activité pendant plusieurs mois. Les fractions précipitées entre 40 et 60 p. 100 de saturation en sulfate d'ammonium entraînent une certaine partie de l'activité phosphatasique, mais c'est la fraction insolubilisée entre 60 et 80 p. 100 qui présente de beaucoup la plus importante activité.

2) *Autres phosphatases acides :* Quand nous avons eu incidemment à comparer le comportement de la phosphatase de la prostate à celui des autres phosphatases acides, nous avons utilisé les préparations phosphatasiques suivantes : la phosphatase des graines de la Moutarde-Blanche et celle du son de Blé purifiées selon notre protocole habituel, décrit précédemment [17], comme représentants des phosphatases végétales du type II ; la takadiastase de la firme Parke, Davis et Co. et un macéré de mycellium de l'*Aspergillus Niger* pour les phosphatases du type III (pH opt. 4,0), et enfin les urines matinales d'homme et de femme soumises à une dialyse de 24 h à la glacière comme source des phosphatases animales du type II.

b) *Détermination de l'activité enzymatique :*

Dans la plupart des essais, nous avons utilisé comme substrat le β-glycérophosphate disodique. Les solutions de ce substrat furent amenées au pH des essais par addition d'acide acétique 2 N. La concentration en substrat dans le milieu était de 0,016 M. Le milieu était tamponné par 5,0 cm³ d'un tampon acide acétique-acétate de sodium (concentration en acétate dans le milieu 0,10 M). Pour un grand nombre d'essais en présence de certains effecteurs, ce pH était de 4,6, l'expérience nous ayant montré qu'à ce pH l'activité de l'enzyme était encore considérable et l'action de l'effecteur en question plus manifeste qu'à pH 5,5, ce qui facilitait l'étude du phénomène. La quantité de la solution enzymatique ajoutée était de 1,0 cm³, comme nous l'avons indiqué précédemment, le volume total de chaque essai était de 50 cm³, la durée d'hydrolyse était de 1 heure à 37° les essais ayant été amenés à cette température avant l'addition de l'enzyme. Dans les cas où nous avons fait varier certains de ces facteurs, les nouvelles conditions adoptées pour ces cas seront précisées sur les Tableaux et les graphiques correspondants.

c) *Courbes d'activité en fonction du pH :*

Le substrat (β-glycérophosphate) fut amené aux pH voulus à l'aide d'acide acétique 2 N. Comme tampons nous avons employé une gam-

me de tampons acide acétique-acétate de sodium 2 M. Pour la zone de pH 5,5 - 6,5, nous avons utilisé le pouvoir tampon du substrat. Nous avons tenu compte des variations de la force ionique et, suivant les recommandations de LUNDQUIST [6], nous avons maintenu la force ionique constante dans tous les essais de chaque série en ajoutant dans les solutions d'acide acétique qui ont servi pour la préparation des tampons une quantité équimoléculaire de ClNa.

Le pH des essais fut contrôlé à l'électrode de verre. Pour les courbes en fonction du pH, en présence de citrate, nous avons utilisé des tampons citrate disodique-acide chlorhydrique et citrate disodique-soude selon SÖRENSEN couvrant la région de pH 2,5 - 6,5 à des quantités variables pour maintenir la concentration en citrate constante sur toute la gamme de pH étudiée. Des précautions ont été prises pour maintenir également constante la force ionique.

Les autres conditions étaient celles exposées en (b).

d) *Etude de l'action des effecteurs :*

Les solutions des substances pures (acides organiques, sels métalliques ou autres effecteurs) amenées au pH des essais à l'aide d'acide acétique ou de soude 2 N ont été ajoutées dans le milieu contenant le substrat et le tampon, avant l'addition de l'enzyme à des quantités convenables pour obtenir la concentration voulue en effecteur. Dans certains cas, une incubation préalable de l'enzyme avec l'effecteur a eu lieu. Dans ces cas, ou quand des précautions particulières étaient nécessaires, ces détails sont précisés dans le texte.

A plusieurs reprises, nous avons eu à doser des petites quantités d'acide citrique (dans des autolysats bruts de la prostate, dans les préparations phosphatasiques dialysées en présence d'acide citrique), dans ces cas nous avons employé la microméthode de WEIL-MALHERBE et BONE [22] avec de petites modifications de détail à la manipulation. L'élimination des matières protéiques fut effectuée par le réactif de FOLIN et WU.

RÉSULTATS.

Action des ions citrate en fonction du pH.

La fig. 1 montre l'influence du pH sur l'activation de la phosphatase de la prostate par les ions citrate. Cette activation, peu considérable aux environs de pH optimum de l'enzyme (pH 5,6) et à des valeurs de pH supérieures à ce dernier voit croître son importance au fur et à mesure que le pH s'abaisse. La phosphatase prostatique montre une activité considérable dans la région de pH 3,6 - 6,0 tandis qu'en l'absence de citrate l'activité baisse vite à partir d'un pH plus acide que 5,0.

La courbe obtenue en présence de citrate a une forme aplatie dans la région de pH 3,5 - 6,0, l'optimum (pH 5,54) est peu saillant (le pourcentage d'hydrolyse du substrat s'élève de 13,8 pour un pH 3,6 à 15,1 pour un pH 5,54). Cette courbe ressemble à celle obtenue avec le phenylphosphate, sans addition de citrate (fig. 2), substrat pour lequel

la phosphatase de la prostate a une affinité plus grande que pour le

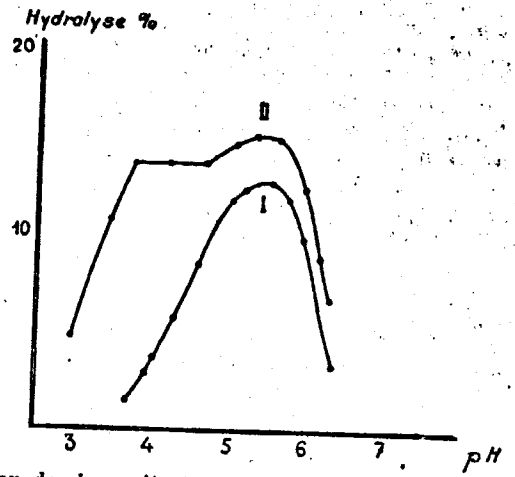

FIG. 1. — Action des ions citrate sur l'hydrolyse du β-glycérophosphate par la phosphatase de la prostate purifiée en fonction du pH.
Concentration en ion citrate dans le milieu 1 × 10^{-2} M :
I. — Sans addition de citrate.
II. — Avec addition de citrate.

β-glycérophosphate et sur l'hydrolyse duquel les ions citrate ont peu d'action (voir également plus loin Tableau IV).

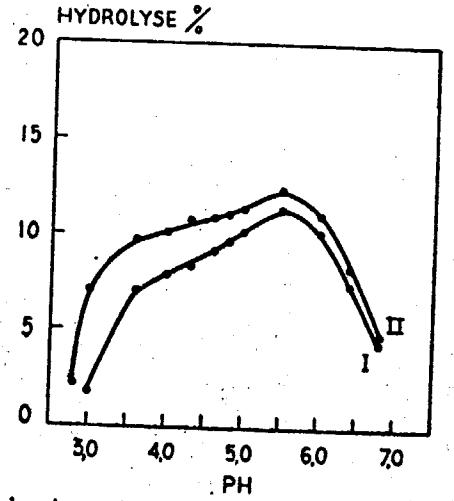

FIG. 2. — Action des ions citrate sur l'hydrolyse du phénylphosphate, en fonction du pH, par la phosphatase de la prostate purifiée.
Concentration en citrate dans le milieu 1 × 10^{-2} M.
Durée d'hydrolyse : 30 minutes :
I. — Témoins, sans addition de citrate.
II. — Essais en présence de citrate.

Par ailleurs, la courbe d'hydrolyse du β-glycérophosphate par la phosphatase prostatique en fonction du pH, en présence des ions citrate rappelle la courbe publiée par KUTSCHER et WÖRNER [3] représentant la stabilité de la phosphatase prostatique en fonction du pH.

Le taux de l'activation de l'enzyme par le citrate à un pH donné (surtout pour des valeurs de pH inférieures à pH 5,0) varie légèrement d'une préparation enzymatique à l'autre. Ceci est probablement en relation avec le degré de dénaturation de l'enzyme. Nous reviendrons plus loin sur ce point.

Le Tableau I indique le pourcentage d'activation (à pH 4,6) par des concentrations variables en citrate que nous avons observé avec la plupart de nos préparations purifiées fraîchement préparées selon notre protocole opératoire, c'est-à-dire avec des enzymes qui ont subi le traitement mentionné plus haut (partie expérimentale (a)).

TABLEAU I.

ACTION DES IONS CITRATE SUR LA PHOSPHATASE DE LA PROSTATE.

Concentration moléculaire en ions citrate dans le milieu	IXIO^{-5}	IXIO^{-4}	IXIO^{-3}	IXIO^{-2}	IXIO^{-1}
Activité relative.....	110,7	146,2	177,3	180,0	157,0

pH 4,6. — Protocole des essais : voir *partie expérimentale* (b).
Les chiffres représentent les activités relatives ; rapport de l'activité de l'enzyme en présence de l'effecteur à celle du témoin sans effecteur, ce rapport étant multiplié par 100.

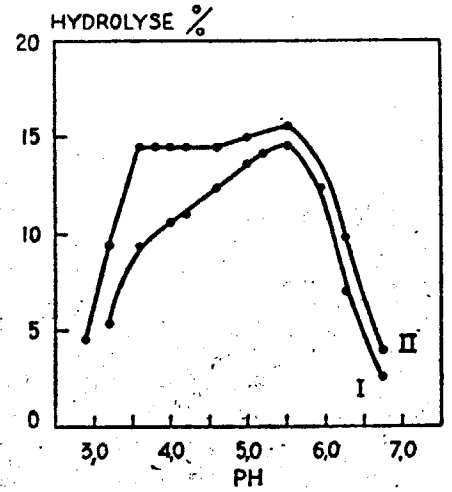

FIG. 3. — Action des ions citrate sur l'hydrolyse du β-glycérophosphate, en fonction du pH, par un autolysat non purifié de prostate humaine.
Concentration en ions citrate dans le milieu : 1 × 10^{-2} M.
I. — Témoins, sans addition de citrate.
II. — Essais avec citrate.

On voit que les ions citrate manifestent leur action à partir d'une faible concentration (10^{-5}M) ; à la concentration 10^{-4}M l'action est déjà considérable la concentration optimale est de 10^{-2}M environ, à des concentrations plus fortes (10^{-1}M) l'activation diminue.

Sur des préparations conservées trop longtemps à la glacière (plus d'un an) et qui ont perdu par vieillissement une partie de leur activité, l'action des ions citrate parait plus forte. Sur des autolysats bruts de la prostate, l'action de citrate est moins marquée que sur les préparations purifiées. Ceci s'explique par le fait que ces autolysats bruts contiennent de petites quantités d'acide citrique, constituant naturel de la sécrétion prostatique.

Nous avons trouvé, dans un autolysat de prostate dilué, une concentration de 8,9 XIO^{-5}M en acide citrique.

La Fig. 3 représente l'hydrolyse du β-glycérophosphate en fonction du pH par un tel autolysat prostatique frais avec et sans addition de citrate.

La courbe obtenue avec l'autolysat sans citrate occupe une position intermédiaire entre la préparation purifiée sans addition de citrate (Fig. 1, courbe I) et les 2 courbes presque identiques, obtenues avec la préparation purifiée ou l'autolysat en présence d'une concentration IXIO^{-2}M en citrate (Fig. 1 et 2, courbes II).

Action de divers composés organiques sur la phosphatase prostatique :

Nous avons recherché si cette action activatrice était spécifique des ions citrate et quelle pouvait être la fonction chimique de ce corps responsable de cette activation.

Dans ce but, nous avons étudié l'action d'un grand nombre de composés sur l'hydrolyse du β-glycérophosphate par la phosphatase de la prostate : acides organiques mono- et di-carboxyliques, acides alcools, acides possédant d'autres fonctions (thiol, amine, cétone), dérivés de l'acide citrique (ester diméthylique de l'acide citrique), polyols, oses.

Le Tableau II montre que, seuls les composés carboxyliques possédant en outre en α- une fonction alcool, thiol ou cétone, sont capables de provoquer l'activation de l'enzyme. C'est ainsi que l'acide lactique et l'acide α-oxybutyrique agissent mais l'acide β-oxypropionique et l'acide β-oxybutyrique n'exercent pas d'activation, l'ester diméthylique de l'acide citrique qui diffère de l'acide citrique par le fait que 2 des carboxyles sont bloqués par estérification, agit comme l'acide citrique. Une fonction thiol ou cétone (cette dernière réagissant probablement sous sa forme énolique) en α confère au produit le même pouvoir d'activation sur la phosphatase de la prostate. Les oses et les polyols étudiés n'ont exercé aucune action.

Les acides α-aminés provoquent une légère inhibition et cette action est due à la fonction amine comme le montrent les essais avec la monoéthylamine et l'hydrazine. Certains acides dicarboxyliques, pos-

sédant un nombre pair d'atomes de carbone (oxalique, succinique, maléique) exercent une certaine inhibition.

TABLEAU II.

ACTION DE CERTAINS ANIONS ORGANIQUES (autres que les citrates) ET QUELQUES AUTRES SUBSTANCES SUR LA PHOSPHATASE DE LA PROSTATE.

Nature de l'effecteur	Activité relative	Nature de l'effecteur	Activité relative
D-malate	180,0	glycolate	160,0
D,L malate	85,2	D-lactate	180,0
D-tartrate	180,0	β-oxypropionate	100,0
L(+)tartrate	4,8	α-oxybutyrate	175,0
D-mucate	173,5	β-oxybutyrate	100,0
D-saccharate	173,8	ester diméthylique de citrate	180,0
Oxalate	70,0	quinate	172,5
Malonate	100,0	pyruvate	150,0
Succinate	84,8	L-cystéine	112,1
Maléinate	81,8	méthylamine	83,4
Fumarate	100,0	hydrazine	75,9
Aconinate	100,0	D-glucose	100,0
Thiomalate	180,0	D-levulose	100,0
L-glutamate	87,5	D-mannitol	100,0
L-aspartate	82,35		

pH 4.6. Tampon acétique. Concentration en effecteur dans le milieu 1×10^{-2} M.
Pour le calcul de l'activité relative, voir Tableau I.

Nous étudierons ultérieurement l'action de l'acide L-tartrique.

Les ions citrate cependant possèdent le pouvoir d'activation sur la phosphatase à un degré bien plus élevé que tous les autres acides α-alcools etc., les carboxyles libres sont nécessaires, comme le montrent les expériences, dont les résultats figurent sur le Tableau III, où l'action de quelques acides α-alcools sur l'enzyme a été étudiée en fonction de la concentration en effecteur.

TABLEAU III.

ACTION DE QUELQUES ACIDES α-ALCOOLS SUR LA PHOSPHATASE DE LA PROSTATE EN FONCTION DE LA CONCENTRATION EN EFFECTEUR.

A Concentration moléculaire en effecteur dans le milieu	Acide		
	D-lactique	glycolique	diméthyl-citrique (ester-diméthylique de l'acide citrique)
$1X10^{-2}$	180,0	160,0	180,0
$1X10^{-3}$	107,0	135,4	127,0
$1X10^{-4}$	102,0	114,7	107,0

pH 4,6. Tampon acétique. Substrat : β-glycérophosphate.
Les chiffres représentent les activités relatives calculées comme pour le Tableau I.

En effet, en comparant les Tableaux I et III nous pouvons observer que les acides glycolique et lactique ont peu d'action à des faibles concentrations, et il en est de même pour l'ester diméthylique de l'acide citrique, par rapport à l'acide citrique.

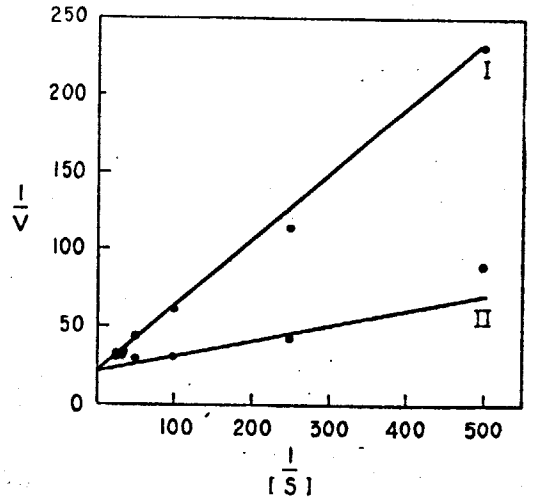

FIG. 4. — Action des ions citrate sur l'affinité K_M de la phosphatase de la prostate pour le β-glycérophosphate.
pH 4,6. I : Essais témoins (sans addition de citrate).
II : Essais effectués en présence de citrate (1×10^{-2} M).

En abscisses : $\frac{1}{[S]}$ = l'inverse de la concentration moléculaire en substrat dans le milieu.

En ordonnées : $\frac{1}{V}$ = l'inverse de la vitesse de l'hydrolyse enzymatique, exprimée en mg de phosphore, libéré sous forme d'acide orthophosphorique par minute.

Cinétique de l'activation par les ions citrate :

Nous avons étudié plus en détail le mécanisme de cette activation par les ions citrate. Dans la Fig. 4 sont représentées suivant la méthode de présentation de LINEWEAVER et BURK [23], les données obtenues en fonction de la concentration en substrat (β-glycérophosphate) avec et sans addition de citrate.

D'après ces expériences, les ions citrate augmentent l'affinité de l'enzyme pour le substrat. L'affinité de la phosphatase prostatique pour le β-glycérophosphate à pH 4,6, calculée d'après ce schéma est de 47,5 en l'absence de citrate et de 145,5 en présence d'une concentration de $1X10^{-2}M$ en citrate. Avec de fortes concentrations en substrat (supérieures à 0,02 M) la vitesse d'hydrolyse en présence de citrate diminue un peu. Ce fait semble indiquer une compétition entre le substrat et l'effecteur pour les mêmes groupements actifs de l'enzyme.

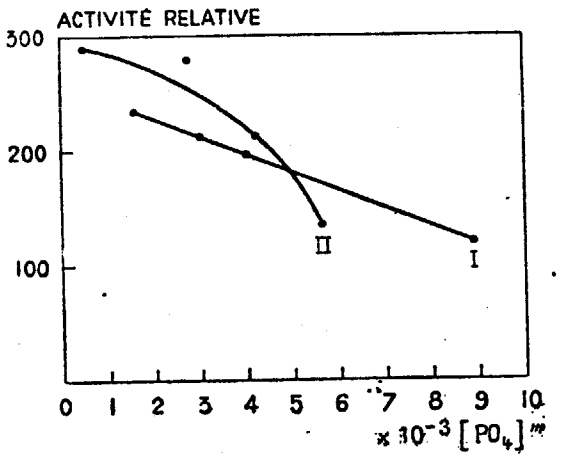

FIG. 5. — Influence des orthophosphates libérés sur la vitesse de l'hydrolyse du β-glycérophosphate par la phosphatase de la prostate en présence des ions citrate.

En abscisses : la concentration moléculaire en orthophosphate $(PO_4)'''$ dans le milieu (phosphates libérés par l'hydrolyse, soit en fonction du temps (I), soit en fonction de la concentration en enzyme (II).

En ordonnées : l'activité relative calculée comme sur le Tableau I, par rapport à des essais témoins sans addition de citrate.
pH 4,6. Concentration en citrate dans le milieu : 1×10^{-2} M.

I : Essais en fonction de la durée d'hydrolyse (concentration en enzyme constante).

II : Essais en fonction de la concentration en enzyme (durée d'hydrolyse constante. Quantité d'enzyme variable : 0,10, 0,50, 1,0, 2,0 cm^3 de préparation enzymatique).

Les essais effectués en fonction de la durée d'hydrolyse et en fonction de la concentration en enzyme (Fig. 5) montrent qu'il existe également une compétition entre les ions citrate et les ions phosphate

libérés. L'enzyme purifié ne semble pas être affecté par de petites quantités de phosphate. Par contre, ces mêmes concentrations en phosphate diminuent l'activation provoquée par le citrate. L'activation plus forte à de faibles taux d'hydrolyse du substrat (125 p. 100 d'activation à 4,3 p. 100 d'hydrolyse du substrat dans l'essai témoin) diminue au fur et à mesure que l'hydrolyse du substrat progresse (20 p. 100) d'activation seulement quand l'hydrolyse est de 47 p. 100 dans l'essai témoin). L'activation citrique dépend, par ailleurs de la concentration en enzyme dans le milieu d'hydrolyse.

Action de citrate sur l'hydrolyse des différents substrats :

Le Tableau IV montre que les ions citrate augmentent la vitesse d'hydrolyse de tous les substrats que nous avons soumis à l'action de la phosphatase de la prostate, mais le taux de l'activation varie suivant le substrat (pour la même concentration moléculaire en substrat et le même pourcentage d'hydrolyse). Ce taux d'activation est en rapport avec l'affinité de l'enzyme pour le substrat en question : l'activation est d'autant plus marquée que l'affinité K_M pour le substrat est plus faible.

TABLEAU IV.

ACTIONS DES IONS CITRATE SUR L'HYDROLYSE DE DIFFÉRENTS SUBSTRATS PAR LA PHOSPHATASE DE LA PROSTATE HUMAINE.

Substrat	Durée d'hydrolyse (minutes)	Activité relative
β-glycérophosphate.....	60	180,0
Phenylphosphate.....	30	116,1
Morpholinoéthanolphosphate.	45	129,6

pH 4,6. Concentration en effecteur : 1×10^{-2} M. La durée du contact de chaque substrat avec l'enzyme a été calculée de façon à avoir une hydrolyse de 10 p. 100 dans les essais témoins (sans effecteur). Les autres conditions sont mentionnées dans la *partie expérimentale* (b).
Activités relatives calculées comme au Tableau I.

Action des ions citrate sur d'autres phosphatases acides :

La phosphatase de la prostate, bien que la plus sensible à l'action des ions citrate, n'est pas la seule phosphatase susceptible d'être activée par cet effecteur. En dehors des phosphatases du type III (takadiastase, *Aspergillus niger*) dont l'activation par les citrates a déjà été étudiée par BAMANN et SALZER [9, 10] et COURTOIS [11], les phosphatases acides végétales présentent cette même propriété bien qu'à un degré beaucoup moins élevé.

Sur le Tableau V, sont comparées les activités de tous ces enzymes en présence de citrate.

TABLEAU V.

ACTION DES IONS CITRATE SUR DIVERSES PHOSPHATASES ACIDES.

A Origine de l'enzyme	B pH de l'essai	C Concentration en enzyme (p. 100)	D Activité relative
Takadiastase (*Aspergillus oryzae*) .	4,0	0,10 g.	201,7
Aspergillus niger	4,0	10 cm³	145,9
Moutarde blanche	4,6	0,02 g.	110,2
Son de Blé	4,6	0,02 g.	114,3

Durée d'hydrolyse : 24 heures pour tous les essais.
Autres conditions opératoires ; voir partie expérimentale (b).
Activités relatives calculées comme pour le Tableau I.

Pour expliquer la nature de l'action des ions citrate sur l'affinité de la phosphatase prostatique pour ses substrats et sur la vitesse d'hydrolyse de ces substrats, nous avons envisagé deux hypothèses :

— L'ion citrate serait soit un « activateur vrai » de l'enzyme soit un « anti-inhibiteur ». Dans le second cas, il agirait en bloquant un inhibiteur présent dans le milieu réactionnel ; il y serait apporté soit par la préparation enzymatique elle-même, soit par les réactifs (substrat, tampon, etc..).

L'utilisation de l'eau bi-distillée préparée dans un appareil de verre pour la préparation de toutes les solutions et de produits purs « pour analyses », et contrôlés par nous aussi pour la présence de certains ions devait, en principe permettre d'éliminer cette dernière éventualité. Restait l'hypothèse d'un inhibiteur naturel existant dans les préparations enzymatiques comme impureté et, comme tels, nous avons envisagé des traces d'éléments métalliques si aisément complexés par des acides α-alcools et présents d'une façon presque régulière dans les cendres des préparations de phosphatase prostatique.

Nous avons pu déceler par spectrographie, dans une préparation purifiée de phosphatase de la prostate, les éléments métalliques suivants :

Mg, Ca, Fe (ce dernier en traces infimes).

Ces métaux solidement adsorbés sur les protéines et, de ce fait non éliminés par dialyse, entraveraient la formation du complexe enzyme-substrat et leur élimination (ou blocage) par le citrate augmenterait l'affinité de l'enzyme pour le substrat.

Dialyse en présence de citrate :

Dans le but de tenter d'élucider ce problème : le citrate agit-il comme un activateur de la phosphatase ou comme un anti-inhibiteur en bloquant un inhibiteur présent dans le milieu ?, nous avons eu recours à des essais de dialyse.

Des solutions de phosphatase prostatique ont été mélangées à volumes égaux avec du tampon citrique M/10 de pH 4,6 et les mélanges

ont été dialysés à +3° contre de l'eau bidistillée, fréquemment renouvelée. L'élimination des ions citrate a été suivie par la recherche qualitative quotidienne et de fréquents microdosages dans le dialysat. La dialyse a été poursuivie encore deux à trois jours après que le dialysat ait cessé de fournir la réaction des citrates selon la microméthode de WEIL-MALHERBE et BONE [21]. Des solutions témoins de phosphatase diluées avec le même volume de tampon acétique de pH 4,6 ont été soumises à une dialyse de même durée.

Au cours de la dialyse, la préparation restant dans le dialyseur fut souvent contrôlée au point de vue de l'activité enzymatique, avec et sans addition de citrate. Sur le Tableau VI sont représentées les principales étapes de cette opération.

TABLEAU VI.

DIALYSE DE LA PHOSPHATASE DE LA PROSTATE EN PRÉSENCE DES IONS CITRATE.

A Durée de la dialyse (jours)	B Concentration moléculaire en ions citrate dans la préparation enzymatique	C Recherche des ions citrate dans le dialysat	D Activité (1)	E Activité en présence des ions citrate 1×10^{-2}M dans le milieu d'hydrolyse (1)	F Activité relative (2)
0	0		1446,6	2598,8	179,6
0	5×10^{-2}		2339,0	—	—
18	$6,2\times10^{-4}$	+	1931,9	2601,6	134,7
40	$2,85\times10^{-5}$	—	1907,0	2584,0	135,4

(1) γ de phosphore minéral libéré sous forme d'acide orthophosphorique par mg d'azote total et par minute dans les conditions standard précisées antérieurement [partie expérimentale (b)]. Hydrolyse du substrat n'excédant pas 20 p. 100.
(2) Calculée comme sur le Tableau I.

La plus grande partie des citrates est éliminée au cours des premiers jours. L'élimination devient ensuite de plus en plus lente. Une petite quantité persiste dans le milieu et elle n'est éliminée que très lentement. Une certaine proportion demeure toujours liée à la préparation enzymatique, même après 46 jours de dialyse lorsque le dialysat concentré n'a plus fourni, depuis plusieurs jours, la réaction de l'acide citrique.

L'activité de la solution enzymatique dialysée en présence des citrates baisse au fur et à mesure que le citrate est éliminé du milieu et l'enzyme peut être réactivé par addition d'une nouvelle quantité de citrate.

Ceci prouverait que les citrates n'agissent pas seulement en éliminant un inhibiteur présent dans le milieu. En effet le pourcentage d'activation par une nouvelle quantité de citrate dépend de la durée de la dialyse, c'est-à-dire de la quantité de citrate encore présent

dans le liquide soumis à la dialyse. Néanmoins, l'activité de l'enzyme dialysé en présence de citrate ne redescend jamais jusqu'à la valeur initiale de l'activité de la phosphatase (c'est-à-dire avant l'addition de citrate). Une certaine activation persiste même après une dialyse très prolongée (44 p. 100 environ d'activation après 40 jours de dialyse). Il paraît logique d'attribuer cette activation aux petites quantités de citrate ($2{,}85 \times 10^{-5}$ M dans la préparation dialysée pendant 40 jours) qui ne franchissent pas la membrane et paraissent être liées à l'enzyme. Il convient cependant de signaler qu'une préparation de phosphatase prostatique purifiée, et ne contenant donc pas de citrate même à l'état de traces, mise en présence d'une quantité de citrate deux fois supérieure à celle trouvée dans les préparations longuement dialysées (ajoutée dans le milieu d'hydrolyse ou incubée avec l'enzyme pendant 8 jours à la glacière) n'a été activée que de 6,5 p. 100. Il est bien possible que l'enzyme dialysé ayant subi un long contact avec une quantité très importante de citrate, a fixé une petite quantité de cet ion, qui est solidement fixée sur l'enzyme et facilite la liaison avec le substrat.

TABLEAU VII.

INFLUENCE DES IONS CITRATE SUR L'INHIBITION DE LA PHOSPHATASE DE LA PROSTATE PAR LES IONS FLUOR.

pH	A Concentration moléculaire en effecteur dans le milieu				
	FNa	Citrate			
		(1) 0	2×10^{-4}	1×10^{-3}	1×10^{-2}
3,6	2×10^{-4}	28,5	—	—	40,5
	1×10^{-3}	4,3	—	—	5,5
4,6	2×10^{-4}	36,4	36,4	65,5	92,5
	1×10^{-3}	0,0	—	—	34,1
5,6	2×10^{-4}	66,7	—	—	98,8
	5×10^{-4}	8,2	—	—	93,7
	1×10^{-3}	5,9	—	—	86,4

Ces expériences (comme celles de la Fig. 6) ont été effectuées sans addition de tampon ; l'inhibition par les ions fluor (à la concentration de 2×10^{-4} M) étant beaucoup plus importante en présence d'une forte concentration en ions dans le milieu, les ions citrate possédant la faculté d'abolir l'inhibition au même degré dans les deux cas. Les solutions du substrat et d'acide citrique ont été amenées au pH voulu par addition de CH_3COOH 2 N ou OHNa 2 N. Les autres conditions opératoires sont celles décrites dans la *partie expérimentale* (b). L'hydrolyse du substrat ne dépassait pas 15 p. 100. Les chiffres représentent les activités relatives calculées comme pour le Tableau I ; celles en absence d'ions citrate (1) par rapport à des témoins ne contenant pas de citrate ; celles des autres colonnes par rapport à des essais témoins contenant du citrate.

Influence des citrates sur l'inhibition de la phosphatase par les fluorures :

Le Tableau VII montre que les ions citrate peuvent soit abolir l'inhibition de la phosphatase de la prostate provoquée par de faibles concentrations des ions fluor (2×10^{-4} ou inférieures), soit diminuer celle provoquée par des concentrations plus élevées en cet inhibiteur. Pour que les ions citrate puissent exercer pleinement cette action, il faut qu'ils existent en grand excès : le rapport fluorure/acide citrique doit être égal à 1 : 50. Enfin cette action dépend du pH du milieu.

La figure 6 montre un exemple de l'action des ions fluor sur la phosphatase prostatique en fonction du pH, en présence et en l'absence des ions citrate.

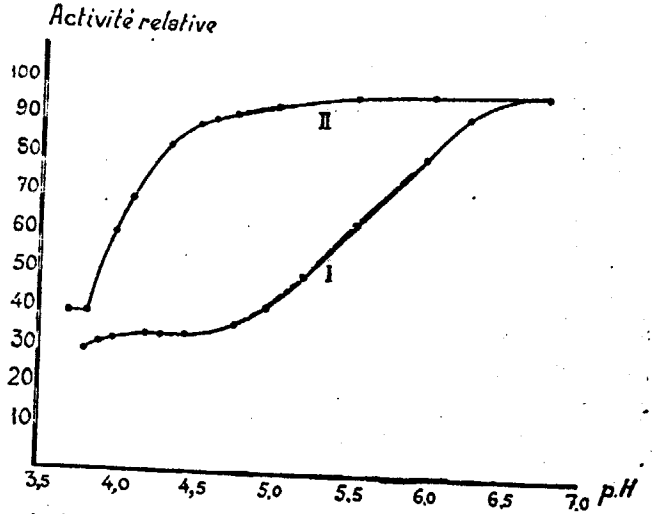

FIG. 6. — Action des ions citrate sur l'inhibition de la phosphatase de la prostate par les ions fluor en fonction du pH.
Concentration en effecteurs : FNa : 2×10^{-4} M.
Citrate : 1×10^{-2} M.
I. Essais sans citrate.
II. Essais avec addition de citrate.
Les activités relatives sont calculées comme mentionné sur le Tableau I : celles de la courbe I par rapport à une série d'essais témoins sans citrate, celles de la courbe II par rapport à des témoins contenant du citrate.
Les expériences ont été effectuées sans addition de tampon comme celles du Tableau VII.

En l'absence de citrate, pour une concentration donnée en ions fluor, l'inhibition a une valeur stable pour toutes les valeurs de pH inférieures à 4,5.

Au-delà de ce pH (4,5), l'inhibition de l'enzyme décroit progressivement. Les ions citrate n'exercent d'autre part, pleinement leur action sur l'inhibition fluorée qu'à des valeurs de pH supérieures à 4,5.

Pour des valeurs moins élevées que ce pH, l'action des citrates devient de plus en plus faible et l'inhibition fluorée s'approche de la valeur obtenue en l'absence de citrate.

Cette action est spécifique des ions citrate et pourrait être attribuée à l'existence de plusieurs carboxyles dans la molécule de l'acide citrique, des autres acides α-alcools etc... qui activent la phosphatase, un certain nombre n'exerce pas du tout cette action sur l'inhibition fluorée et les autres l'exercent mais à un degré très inférieur à celui de l'acide citrique ; le blocage de deux des carboxyles de l'acide citrique (ester diméthylique de l'acide citrique) entraine une forte diminution de cette action (Tableau VIII).

TABLEAU VIII.

INFLUENCE DE DIFFÉRENTS ANIONS ORGANIQUES SUR L'INHIBITION DE LA PHOSPHATASE DE LA PROSTATE PAR LES IONS FLUOR.

A B C

Nature de l'anion organique	Concentration moléculaire en anion organique	Concentration moléculaire en ions fluor dans le milieu	
		2×10^{-4}	1×10^{-3}
Aucun	—	36,4	0,0
Citrate.....	1×10^{-2}	92,5	34,1
D-malate.....	1×10^{-3}	41,7	—
D-malate.....	1×10^{-2}	48,4	—
Diméthyl-citrate.....	1×10^{-2}	56,0	0,0
Aconitate.....	1×10^{-2}	36,4	0,0
Lactate.....	1×10^{-2}.	36,4	0,0

pH 4,6. Sans addition de tampon.
Conditions expérimentales décrites dans la partie expérimentale (b). Les chiffres représentent les activités relatives calculées comme au Tableau I.

Les citrates n'influencent pas l'inhibition des phosphatases végétales par les ions fluor.

DISCUSSION GÉNÉRALE DES RÉSULTATS.

Nous avons discuté plus haut, à propos de chaque tableau ou figure, les faits apportés par ces expériences. De l'ensemble de ces résultats, les faits suivants se dégagent :

1) Les acides organiques possèdant en α- une fonction alcool, thiol ou cétone, augmentent l'affinité de la phosphatase de la prostate humaine (et de certaines autres phosphatases acides) pour ses substrats. L'existence de plusieurs carboxyles dans la molécule accentue cette

propriété des acides α-alcools etc... et l'acide citrique avec ses 3 carboxyles se montre le plus efficace de tous les acides étudiés. Cette action est probablement, pour une grande partie, une action anti-inhibitrice : élimination d'un inhibiteur présent, à l'état de traces, dans les préparations enzymatiques même les plus purifiées qui serait en partie libre dans le milieu (dissocié et susceptible d'être éliminé par dialyse en présence des ions citrate) et en partie solidement fixé sur l'enzyme ou même faisant partie de la molécule enzymatique. Nous ne pouvons pas encore nous prononcer sur la nature de cet inhibiteur. Les éléments métalliques (Ca, Mg, Fe) présents en quantités minimes dans les cendres des préparations enzymatiques utilisées peuvent probablement être la cause de ce phénomène.

2) Les essais effectués sur des préparations purifiées de phosphatase de la prostate en utilisant le phénylphosphate comme substrat et les essais avec d'autres substrats en présence des ions citrate mettent en évidence le fait que cet enzyme présente une activité dans une zone de pH plutôt étendue (pH 3,6 — 6,2) ; avec un optimum très peu prononcé (vers pH 5,54).

Ce fait est intéressant du point de vue de l'action physiologique de la phosphatase de la prostate. Cette phosphatase peut être particulièrement active dans le milieu vaginal et subir sans diminution appréciable de son activité les variations de pH de ce milieu (pH 4,0 — 5,6) comme l'avaient précédemment signalé KUTSCHER et WÖRNER [3], à propos de leurs essais sur l'inactivation thermique de l'enzyme en fonction du pH. La présence de quantités importantes de citrate dans le sperme garantit la stabilité et l'activité de l'enzyme dans cette zone de pH.

3) L'action des ions citrate sur l'inhibition de la phosphatase de la prostate par les ions fluor mérite d'être discutée plus en détail. Nous ne pouvons pas encore affirmer qu'il existe ou non une relation entre l'action activatrice ou anti-inhibitrice des ions citrate et leur pouvoir d'abolir (ou de diminuer, suivant le cas) l'inhibition provoquée par les fluorures. Nous avons pourtant indiqué que les ions citrate sont les seuls, parmi les anions organiques étudiés, à posséder ce pouvoir à un degré élevé.

D'autre part, l'existence de plusieurs carboxyles dans la molécule de l'acide citrique semble être la cause de cet état de choses. Les autres acides α-alcools etc... ont peu d'action sur l'inhibition fluorée.

Le citrate est, nous l'avons déjà vu, l'ion qui possède, parmi tous les anions étudiés, au plus haut degré le pouvoir activateur ou anti-inhibiteur sur la phosphatase. Il se pourrait bien que les ions fluor forment avec l'inhibiteur contenu dans le milieu, un complexe qui a un pouvoir inhibiteur plus élevé que les ions fluor seuls. Les ions citrate en se combinant à l'inhibiteur et en l'éliminant du milieu réduiraient l'inhibition de l'enzyme par les ions fluor à sa valeur vraie : combinaison enzyme + fluorure et non plus enzyme + inhibiteur + fluorure. L'inhibiteur inconnu aurait plus d'affinité pour les ions fluor que pour les ions citrate, comme on peut s'en rendre compte du fait

qu'il faut toujours un grand excès de citrate pour abolir (ou diminuer) l'inhibition exercée par les ions fluor.

La courbe I de la figure 6 représenterait la dissociation en fonction du pH du complexe enzyme + fluorure (ou enzyme + inhibiteur + fluorure) ou celle du complexe fluorure + inhibiteur inconnu.

Un fait intéressant est que la valeur de pH à partir de laquelle l'inhibition exercée par les ions fluor commence à diminuer (sans addition de citrate) est le pH 4,4, ce qui correspond à la valeur trouvée par KUTSCHER et PANY [4] pour le point isoélectrique de l'enzyme prostatique.

Ce fait signifierait que si la courbe représente la dissociation du complexe enzyme + fluorure en fonction du pH, ce complexe est plus stable du côté acide du point isoélectrique de l'enzyme.

L'inhibition par les ions fluor a une valeur fixe pour chaque concentration en ions fluor pour toutes les valeurs de pH plus acides que le point isoélectrique de la phosphatase. A partir de ce pH, le complexe commence à se dissocier et l'inhibition diminue. C'est le contraire pour les ions citrate : la combinaison citrate + inhibiteur inconnu ou citrate + enzyme qui protège la phosphatase des ions fluor, est plus stable du côté alcalin de cette même valeur de pH (pH 4,4 — 4,5) ; pour des valeurs de pH plus acides, cette combinaison se dissocie et les ions citrate sont de moins en moins efficaces pour la protection de l'enzyme contre les ions fluor. Or, nos expériences de dialyse de la phosphatase de la prostate en présence de citrate, exposées plus haut, tendent à prouver que l'inhibiteur naturel (probablement un ion métallique) est peu dissociable et semble faire partie de la molécule enzymatique. Cet inhibiteur gênerait la combinaison enzyme — substrat et ceci aurait lieu surtout du côté acide du pH optimum de l'enzyme. La combinaison des ions fluor à cet inhibiteur interdirait l'accès de l'enzyme au substrat.

Il faudrait admettre que les ions citrate complexent cet inhibiteur sur la surface de l'enzyme et, simultanément, et d'une façon indépendante, favorisent la formation de la combinaison enzyme-substrat. Ceci expliquerait l'action activatrice (ou anti-inhibitrice) des ions citrate et en même temps leur effet dans le cas de l'inhibition fluorée.

Il existerait une compétition entre les ions citrate et les ions fluor qui serait influencée par le pH et les concentrations relatives en ions citrate et en ions fluor.

Nous désirons remercier MM. les Professeurs P. FLEURY et J. E. COURTOIS pour l'intérêt qu'ils ont porté à ce travail.

RÉSUMÉ.

1) L'action de nombreux anions organiques a été étudiée sur la phosphatase de la prostate humaine purifiée. Les acides possédant en α- une fonction alcool, thiol ou cétone, augmentent l'affinité de cet enzyme pour ses substrats. Les ions citrate présentent cette action à un degré plus élevé que les autres ions étudiés.

L'activation est plus manifeste du côté acide du pH optimum de la phosphatase. La courbe de l'affinité de l'enzyme en fonction du pH a, en présence des ions citrate, une forme aplatie dans la zone de pH 3,6 — 6,2 et présente un optimum peu prononcé vers pH 5,5 — 5,6.

2) Les ions citrate ont en plus le pouvoir d'abolir l'inhibition de l'enzyme par des faibles concentrations des ions fluor et de diminuer celle provoquée par de fortes concentrations du même ion. Cette action est fonction du pH.

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§ 8. Höll apotekaren, fil. lic. T. Canbäck föredrag över ämnet: »Reaktionen mellan aromatiska nitroföreningar och aktiva metylengrupper samt några av dess analytiska tillämpningar». Med anledning av föredraget yttrade sig herrar Erdtman, Lindberg, Vestin, Ölander och föredragshållaren.

On the metabolic acetylations.

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Introduction.

The metabolic acetylation of amino groups foreign to the organism, particularly of arylamines, is a well-established fact. This reaction has gained importance especially because the bacteriostatic action of the sulphonamides has been shown to depend upon the biochemical displacement between sulphonamides and *p*-aminobenzoic acid (cf. only 46). The acetylation thus plays an essential rôle in the inactivation of sulphonamides. The extent of acetylation, as well as certain toxic symptoms in the clinical use of the sulphonamides, also indicate that acetylation may be associated with certain central physiological functions of the organism. Thus, *James* (27) suggests that the injurious effects of sulphanilamide and sulphapyridine may be due chiefly to the sudden withdrawal from the body of acetyl-precursors in an attempt to detoxicate the drug by synthesis. The administration of acetylsulphanilamide as such would not make this sudden demand but may well be injurious in other ways.

Synthesis of citric acid is one of the most fundamental physiological functions of the living organism. Recently, *Martius* (59) has modified the conception of the mechanism of citric acid formation which he had advanced, together with *Knoop*, some time earlier (for review cf. 58). According to this view, citric acid would not be formed through a ketol condensation from pyruvic and oxalacetic acids, but the first phase of the synthesis would be a dehydrogenation of pyruvic acid, followed by decarboxylation. The ketene-type radical produced would then, coupling with oxalacetic acid, give rise to the formation of citric acid. *Martius* suggests that this dehydrogenation should in every case be regarded as the first phase in the dissimilation of pyruvic acid in organism. In the same connection he therefore refers to the possibility that this radical, functioning in the citric acid synthesis, might play an active part also in the formation of diacetyl (acetylmethylcarbinol) and in the acetylation reactions.

The rôle of diphosphothiamine in the dehydrogenation of pyruvic

acid has become apparent already from *Lipmann*'s work. It is true that he assumed the action of diphosphothiamine in this case to be associated with the reversible reduction-oxidation of the molecule, a view which, however, was proved to be untenable by the experiments of *Stern* and *Melnick* (75; cf. also 4).[1] The problem now seems to have received its solution by the recent work of *Myrbäck et al.* (62). By showing that diphosphothiamine occurs *in vivo* also oxidized to disulphide, as diphosphothiamine-SS, they could prove that a redox-system of the well-known type like cysteine-cystine or GSH-GSSG is indeed possible with thiamine, as suggested already by *Zima et al.* (87) with regard to the animal organism.[2]

Concerning the formation of diacetyl (acetylmethylcarbinol) we have earlier shown (*Suomalainen* and *Jännes* 78) that a radical acting like a ketene most probably exists *in vivo* and that its formation is evidently catalyzed by the diphosphothiamine-SS redox-system of *Myrbäck*. Since *Martius*' own investigations already suggest that this radical functions in the formation of citric acid, we have made an attempt here to ascertain its rôle in the acetylations occurring in the living organism. If such rôle can be established, then these three processes — *viz.* formation of citric acid, diacetyl (with homologues) and of acetylamines — would, in fact, be merely different manifestations of the same reaction system: the metabolic acetylation. The course of the reaction would then be largely determined by the component which acts as an acceptor, whether oxalacetic acid, (acet)aldehyde or (aryl)amine.

In regard to acetylation this assumption becomes increasingly probable in view of certain purely chemical model reactions. Thus, pyruvic acid has been shown to acetylate choline *in vitro* in the presence of an oxidizing agent (1). And synthetic ketene has been used for acetylation in several instances, recently even on an industrial scale. As *Cahill* and *Burton* (13) state:

»Although it reacts with water to form acetic acid, ketene combines preferentially with the α-amino group of an amino acid dissolved in water, and it was shown by Bergmann and Stern that amino acids may be conveniently acetylated with this reagent. Because of the simplicity and effectiveness of the reaction, ketene has also been used to acetylate such physiologically active proteins as pepsin and insulin.«[3].

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[1] Moreover, *Karrer et al.* (31) have recently shown that thiamine by reduction with sodium dithionite does not form a dihydroderivative but is reductively split with the liberation of 4-methyl-5-oxyethyl-thiazole.

[2] The fact that, according to *Karrer* and *Viscontini* (32), diphosphothiamine disulphide is inactive as cocarboxylase, does not exclude the possibility that it functions in the dehydrogenation of pyruvic acid. — The cocarboxylase effect of the thiol form of diphosphothiamine should be noted as an interesting feature

[3] It is very interesting that — as reported quite recently by *Nath* and *Brahmachari* (63) — »the intermediary fat metabolism products, particularly the keto-acids and esters [pyruvic acid and acetoacetic acid], are responsible for causing partial or complete inactivation of insulin and sometimes give rise to what is known as 'insulin-refractory' cases. It has further been shown that such inactivation takes place *in vitro* as well as *in vivo*.« (Cf. pag. 317.)

In addition, *Fishman* and *Cohn*'s (20) work with D_2O, and *Bernhard* and *Steinhauser*'s (8) as well as *Bloch* and *Borek*'s (8 a) with deuterioacetic acid, suggest that the acetylation of amino acids in organism also follows, at least partly, the same course as that of arylamines.

The acetylation of sulphonamides will be discussed by one of us in another publication (*Kinnunen* 31), from which the following experimental results are taken.

Experimental.

Effect of thiamine on the acetylation of arylamines in vivo.

In some *in vitro*-experiments with rabbit liver, carried out last winter (1945–46), the acetylating power of the preparations was found to be surprisingly low (*Kinnunen*). Since, in view of what is stated above, this could be assumed to be a result of a thiamin deficiency in the diet of the animals, the effect of B_1-injections on the acetylation of sulphapyridine *in vivo* was studied with animals on a corresponding diet.

It has long been known that the acetylating power varies greatly in different individuals. Thus, *Klein* and *Harris* (35) and *Martin et al.* (57) have paid attention to this phenomenon also in regard to sulphonamides without, however, being able to find an explanation for it. That thiamine might be associated with acetylation has, it is true, been suggested earlier (28), and lately *Martin* and *Rennebaum* (56) have also found that acetylation of sulphanilamide is »defective in rats with thiamin or riboflavin deficiency». *Lipmann* (52) has also recently established increased acetylation by addition of cocarboxylase, in liver preparations of thiamine deficient pigeons. He interpretes this, however, so that »in the thiamine-deficient system the process of conjugation rather than the availability of the acetyl group is inhibited».

Rabbits were kept on a diet consisting chiefly of oats and hay. 0.5 g of sulphapyridine powder was administered daily with the food. In order to explain the effect of thiamine, daily 25 mgs thiamine hydrochloride (»*Medica*») were injected subcutaneously in part of the animals. — It may be noted that the diphosphothiamine content of an avitaminotic organism has been found to attain its normal level remarkably rapidly — in less than half an hour — after injection of thiamine (64, 83).

The urine of two animals was collected jointly. Acetylated and total sulphapyridine were determined on the combined sample by *Kimmig*'s (33) modification of the thymol method.

Table 1 shows the results of an experiment with four rabbits, two of which were given thiamine injections. The initial acetylating power of all the animals was on the same level.

Table 1. Effect of thiamine on the acetylation of sulphapyridine in rabbits *in vivo*.

	Acetylated sulphapyridine, % of total							
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day
Controls	30.0	29.0	30.8	14.3	12.4	19.0	27.5	11.1
B_1-injected	59.2	50.0	68.4	50.0	59.7	80.0	69.4	76.2

Acetylation of arylamines as a branch of the metabolic acetylation.

It was mentioned above that acetylation of arylamines may be regarded as a branch of metabolic acetylation. On this assumption it could be expected that the addition of other acceptors of the acetylating component would have a decreasing effect on acetylation of amines. Thus, acetaldehyde would be expected to function primarily, not as an acetylating compound, but as an acceptor, which is converted into diacetyl and further into acetylmethylcarbinol. Furthermore, *Green et al.* (21) have shown that propionaldehyde is converted into propionylmethylcarbinol (or its isomer acetylethylcarbinol) by an enzyme preparation from pig heart. That an addition of acetaldehyde does not, in fact, increase the acetylation of arylamines is evident already from *Hensel*'s (25) work. It may be mentioned as an interesting feature in this connection that acetaldehyde, in contrast to pyruvic acid (69), does not increase the yield of citric acid either — as shown by *Simola et al.* (for review cf. 22).

It naturally follows that also oxalacetic acid, as precursor of the tricarboxylic acid cycle, would function analogously as acceptor to the acetylating component. For practical reason we have employed in our experiments, instead of oxalacetic acid, its precursor malic acid, an addition of which, especially together with pyruvic acid, was found by *Simola et al.* (for review cf. 22) to increase strongly the formation of citric acid *in vitro*.

The *in vitro*-experiments with rabbit liver were carried out mainly according to *Klein* and *Harris* (35).

3 g (fresh weight) of minced rabbit liver was added into 17 ml Ringer-bicarbonate solution containing 0.2 per cent glucose and 2 mg sulphapyridine. The substance to be tested was present equimolar to sulphapyridine, the acetylating substrate again having a 10-fold molar concentration.

Oxygen and nitrogen were used without a CO_2-admixture; the nitrogen was freed from traces of oxygen by yellow phosphorus.

A detailed report of the experimental methods will be published elsewhere (34).

The ratio of acetylated to total sulphapyridine was determined after finished experiment from the trichloroacetic acid filtrate as above.

The results of one series of experiments are given in Table 2.

Table 2. The effect of substances, acting as acceptors to the acetylating component, on the acetylation of sulphapyridine by rabbit liver aerobically *in vitro*.

Substrate	Added substance	Acetylated sulphapyridine, % of total		
		1 hr	2 hrs	4 hrs
Control	—	16.2	18.4	28.1
Sodium acetate	—	18.9	23.7	34.2
" "	Acetaldehyde	11.7	15.4	25.0
" "	Sodium malate	8.9	18.3	26.1
" pyruvate	—	17.9	21.2	27.5
" "	Acetaldehyde	14.0	19.2	23.4
" "	Sodium malate	11.0	16.2	20.0

An analogous series of experiments (Table 3) again shows that addition of sulphapyridine has an inhibiting effect on the formation of citric acid.

Citric acid was determined from the trichloroacetic acid filtrate according to *Pucher, Sherman* and *Vickery* (66; cf. also 42).

Table 3. Effect of sulphapyridine on citric acid formation by rabbit liver aerobically *in vitro*.

Substrate	Added substance	Citric acid, γ per 1 g (fresh weight)		
		15 mins	30 mins	60 mins
Control.....	—	40	31	31
Sodium pyruvate.....	—	94	72	45
» »	Sulphapyridine	62	45	40

The inhibiting effect of aldehydes again seems to be due — not to the accepting of the acetylating component, with the formation of diacetyl or homologues — but to the well-known *in vitro*-reaction between arylamines and aldehydes, *i. e.* to the formation of *Schiff*'s bases:

$$R \cdot CHO + H_2N \cdot Ar \longrightarrow R \cdot CH : N \cdot Ar + H_2O.$$

This possibility has been proposed by *v. Euler et al.* (17) in regard to yeast. We shall revert to this problem in another connection.

The influence of acceptance of hydrogen on anaerobic acetylation.

We have assumed above that the function of *Myrbäck*'s diphosphothiamine-SS redox-system would be a prerequisite to the formation of the acetylating component from the carbohydrate derivatives. This assumption is in agreement with the fact that aerobicity favours the acetylation process — and also the citric acid formation. Thus, *Klein* and *Harris* (35), who, indeed, regard acetate as the acetylating component, write:

»There is little anaerobic acetylation compared with the acetylation in aerobic controls. The addition of acetate causes a very marked increase in the extent of the anaerobic conversion. ... The decrease in acetylation under anaerobic conditions is, therefore, mainly due to the anaerobic inhibition of acetate formation.«

It would therefore be expected that substrates like pyruvic acid and acetaldehyde, which themselves may function as hydrogen acceptors, would acetylate relatively more strongly under anaerobic conditions than aerobically, in comparison to substrates which, like acetic acid, cannot accept hydrogen. From this standpoint it is interesting to note *Klein* and *Harris*' report that »pyruvate and acetaldehyde give [relatively] good effects anaerobically», although their interpretation of the question is hardly the right one.

In order to shed light on this problem parallel experiments on the acetylation of sulphapyridine *in vitro* were made in oxygen and nitrogen atmospheres. Results

of an anaerobic experiment are given in Table 4. This experiment was analogous to the one illustrated by Table 2.

Table 4. Acetylation of sulphapyridine by rabbit liver anaerobically *in vitro*.

Substrate	Acetylated sulphapyridine, % of total		
	1 hr	2 hrs	4 hrs
Control	12.0	12.7	15.0
Sodium acetate	13.9	15.0	16.7
» pyruvate	16.7	18.9	21.7

Acetaldehyde which distinctly inhibited aerobic acetylation, also when added alone, caused in this anaerobic experiment an equally extensive acetylation as acetate; sulphapyridine acetylated 16.7 % in 4 hrs.

Discussion.

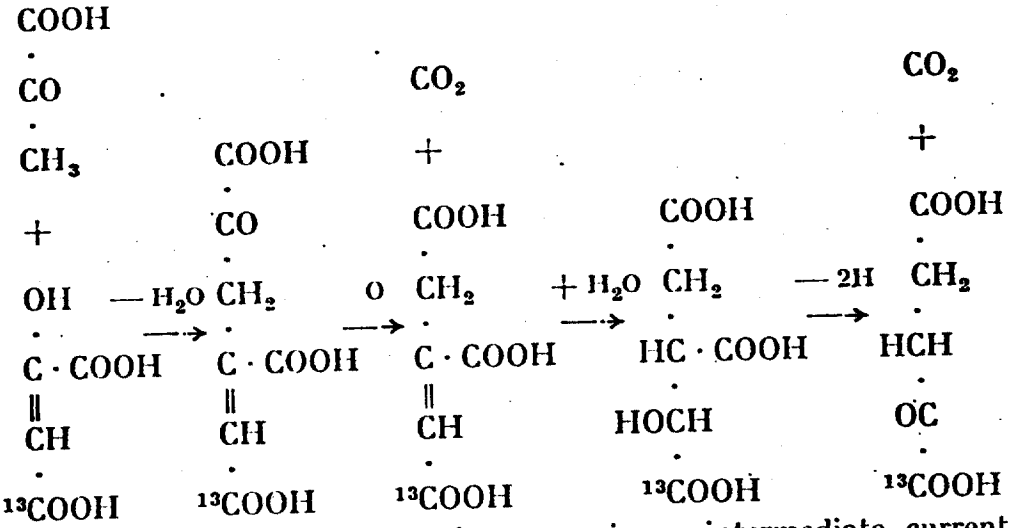
It should be borne in mind that thiamine seems to play an important rôle also in the citric acid synthesis, as suggested by *Simola*'s school already in 1939 (23; cf. also 70). This view has later received confirmation from different quarters (72, 3, 5).

Knoop and *Martius*' original assumption that the condensation of pyruvic and oxalacetic acids is the primary phase in the tricarboxylic acid cycle, has been included also in the newest modification of the *Krebs*' cycle (39). However, it is difficult to interprete the established rôle of thiamine in the citric acid synthesis on the basis of this assumption. Thus, *Krebs* (41, 38; cf. also 71) supports his view of the rôle of thiamine solely by its catalytic influence on the carboxylation of pyruvic acid into oxalacetic acid. However, this assumption cannot explain satisfactorily the fact that the dissimilation of pyruvic acid has been found to proceed at a much higher rate in the presence of O_2 than in its absence (2, 41). The carboxylation of pyruvic acid ought to proceed anaerobically as well as aerobically and the condensation to the tricarboxylic acid stage should likewise be possible also anaerobically. It is further interesting to note that, according to *Werkman et al.* (36, 30; cf. also 81), the enzyme preparation which catalyzes the formation of oxalacetate from pyruvate and bicarbonate — also under anaerobic conditions — requires the addition of manganese salts but not cocarboxylase or inorganic phosphate.

Krebs' assumption has been subjected to criticism also by *Barron*'s school (5). They point out in particular that — according to the theory of *Krebs* — the utilization of pyruvate in the presence of malate ought to be independent of the presence of thiamine, since the product of thiamine catalysis, oxalacetate, was already supplied. In the presence of pyruvate and malate addition of thiamine to the kidney of avitaminotic rats called, however, a 7-fold increase in the utilization of pyruvate (5).

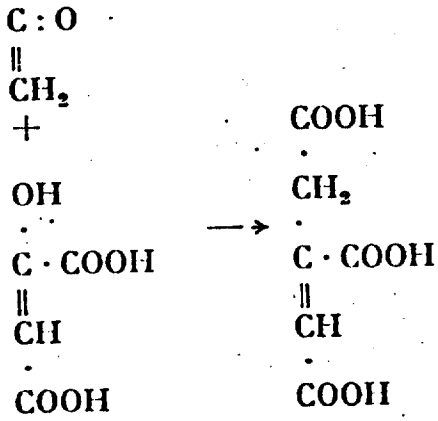
On the basis of *Myrbäck*'s diphosphothiamine-SS redox-system the

decisive rôle of thiamine in the citric acid synthesis would, on the other hand, receive its natural explanation in the radical theory of *Martius*. The isotope research of recent years has shown, however, that citric acid cannot be an intermediate in the tricarboxylic acid cycle, which view is still held by *Martius*. Thus, already *Sonderhoff* and *Thomas* (73) found that dissimilation of $CD_3 \cdot COOH$ with yeast leads to succinic acid $HOOC \cdot CH_2 \cdot CD_2 \cdot COOH$. *Evans, Jr.* and *Slotin* (19), on one hand, and *Wood et al.* (86), on the other, have shown that the modifications of the »physiological« oxalacetic acid, $HOOC \cdot CO \cdot CH_2 \cdot {}^{14}COOH$ and $HOOC \cdot CO \cdot CH_2 \cdot {}^{13}COOH$, lead to α-ketoglutaric acid containing the isotopic carbon entirely in the carboxyl proximal to the keto group (cf. also 12). These findings inevitably lead to the conclusion that the primary »condensation« product must have an unsymmetrical molecular structure »for, if α-ketoglutarate was derived from a symmetrical molecule, the fixed carbon would be equally distributed in the two carboxyl groups« (86). *Wood et al.* propose the following scheme in the case of ^{13}C:



Citric acid being thus out of question as a primary intermediate, current theories are in favour of *cis*-aconitic acid which may further convert into citric acid or — directly — into *iso*citric acid.

According to *Martius* the fusion of the ketene radical with oxalacetic acid ought to lead to the formation of citric acid lactone. But, in our opinion, the fusion may be able to produce *cis*-aconitic acid directly:



And this possibility would also bring the radical theory into agreement with the known facts.

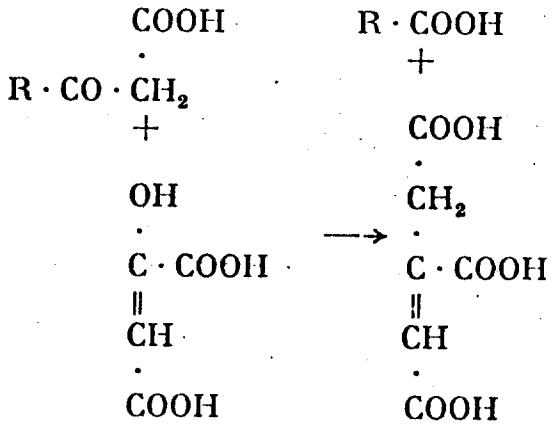
Recently also American workers have come to a corresponding conclusion; thus, *e. g. Lardy* and *Elvehjem* (47) write:

»The key substance in the [tricarboxylic acid] cycle is the two-carbon compound which condenses with oxaloacetate and, in accordance with the most recent evidence, is here designated as an 'activated' acetyl radical [$CH_3 \cdot CO \cdot X$]. . . . Cocarboxylase is necessary for the conversion of pyruvate to the two-carbon compound and hence for the conversion of carbohydrate to fat.

In normal metabolism the acetyl compound is rapidly oxidized, presumably after coupling with oxaloacetate, through the isocitric acid cycle.«

From the viewpoint of the radical theory it is interesting to note that pyruvate aerobically is converted by liver into acetaldehyde (14) and that also the »dismutation« of pyruvic acid depends on thiamine as shown particularly by *Lipmann* (for review cf. 5; also 37, 26, 76).

The radical theory would thus lead from pyruvate to the tricarboxylic acid cycle. But, as shown years ago by *Simola* (68; cf. also 70), both butyric acid and the β-keto acids are also metabolized by way of the tricarboxylic acid cycle. This finding has likewise only recently been confirmed by other workers, *Wieland* (85), *Martius* (59) and *Breusch* (11) having arrived almost simultaneously at this conclusion which, besides, *Buchanan et al.* (12) have confirmed with the use of acetoacetic acid marked with ^{13}C in the carboxyl and β-carbon positions (cf. also 26 a):



According to *Martius* there exist two different enzyme systems capable of forming citric acid, one from pyruvic acid, the other from β-keto acids through a non-hydrolytic cleavage. These two enzymes, he says, occur side by side but in different quantities in almost all organs.

Since the liver tissue does not oxidize acetoacetic acid, *Lehninger* (50; cf. also 61) quite recently has succeeded in distinguishing these two metabolic pathways in the oxidation of both pyruvic acid and fatty acids by rat liver suspensions. Thus, in the absence of oxalacetate they are both metabolized with the formation of acetoacetic acid, but in the presence of oxalacetate or its precursors their metabolism

proceeds directly through the tricarboxylic acid cycle. So, the course of oxidation is determined by the supply of oxalacetate or its precursors, the ketone bodies being formed in tissues lacking in oxalacetate.

This view would also provide a simple explanation for the occurrence of acetone bodies as metabolites as a result of disturbed carbohydrate metabolism, *i. e.* under conditions of fasting or *diabetes mellitus* (cf. *e. g.* 50). *Krebs* (40) suggests that insulin functions as a catalyst of the citric acid cycle. It is interesting to observe that insulin may accelerate also the acetylation process. So, the acetylation of *p*-aminobenzoic acid in the rabbit is increased by the injection of insulin, but no increase is obtained by the simultaneous injection of insulin and reduced glutathione (24; cf. however 57).

Recent isotope research (cf. only 78 a, 82 a) has also shown definitely that — as suggested already by *Loeb* (51) — acetic acid *in vivo* is condensed to acetoacetate. The path of acetic acid into the acetylating compound stage thus seems clear, which means that also acetate, *via* acetoacetate, can enter the tricarboxylic acid cycle by condensation with oxalacetate, and acetylate aromatic amino groups, as suggested by *Bernhard* (6, 8), by *Bloch* and *Rittenberg* (10) and in regard to sulphonamides especially by *Lipmann* (52). This view is supported also by the fact that *Bloch* and *Rittenberg* (9), working with isotopes, proved that both halves of butyric acid are employed in forming acetyl groups.

The formation of acetoacetate from pyruvic acid has been ascribed to the condensation of pyruvic acid with acetate (cf. only 16, 18); we refer here particularly to *Krebs* (44; cf. however 82 a) who regards acetopyruvic acid as an intermediate of this condensation:

$$CH_3 \cdot COOH + CH_3 \cdot CO \cdot COOH \longrightarrow CH_3 \cdot CO \cdot CH_2 \cdot CO \cdot COOH.$$

The rôle of thiamine in the formation of acetoacetic acid (41, 5) would, however, be more easily understood on the basis of the ketene-like radical, produced from pyruvic acid through dehydrogenation:

$$CH_3 \cdot COOH + \begin{matrix} CH_2 \cdot C : O \\ | \quad | \end{matrix} \longrightarrow CH_3 \cdot CO \cdot CH_2 \cdot COOH.$$

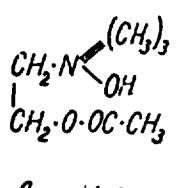
By administration of deuterio-alcohol *Bernhard* (7) has shown that also ethyl alcohol may function as acetylating component *in vivo*. He therefore concludes that alcohol in the organism is metabolized *via* acetic acid. Later, however, he states (8): »Durch Alkohol-Verbrennung entstehende Essigsäure reagiert leichter als verfütterte, es wird eine bedeutend D-reichere Acetylverbindung erhalten.» These apparently contradictory statements (cf. also 9) are explained easily, however, if the opposite hypothesis is accepted, *viz.* that the dissimilation of alcohol in the organism does not proceed over acetic acid. Through dehydrogenation alcohol is, indeed, converted to acetaldehyde, further dehydrogenation leading probably again to the ketene radical. True, this radical might add water and be thus converted to acetic acid, but it might also participate in the general metabolic reactions as such.

— With regard to the formation of acetoin from alcohol in organism reference is made to a review by *Westerfeld* and *Doisy, Jr.* (84).

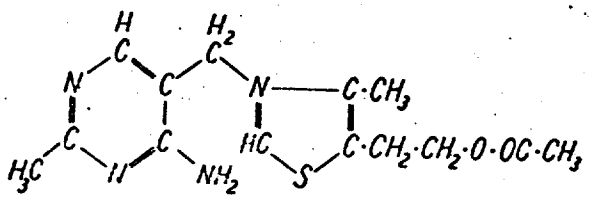
$$CH_3 \cdot CH_2OH \xrightarrow{-2H} CH_3 \cdot CHO \xrightarrow{-2H} CH_2 \cdot C:O \left[\xrightarrow{+H_2O} CH_3 \cdot COOH \right]$$

It is interesting to note that also the acetylation of choline — the formation of acetylcholine — is *in vivo* catalyzed by thiamine (55). Moreover, according to *Torda* and *Wolff* (79, 79 a), »the enzyme involved in the acetylcholine synthesis contains an active —SH group, since the process is inhibited by agents that decrease the activity of the —SH group (monoiodoacetate, alloxan, unsaturated ketones, oxidizing agents, penicillin) and increased by agents protecting the activity of the —SH group (glutathione, cysteine, antioxidants, sodium pyrophosphate in low concentrations)».

Why diphosphothiamine, contrary to other primary amines, does not itself act as acceptor to the acetyl group, is explained by *Stern* and *Melnick*'s work (74). Using ketene they could show *in vitro* that the amino group in free thiamine is resistent against acetylation. On other hand, the hydroxy group of the alcohol side chain — which is phosphorylated in diphosphothiamine — has been acetylated as in choline. Thus these two substances, acetylcholine and acetylthiamine, are indeed split by the same enzyme, cholinesterase (60, 77).

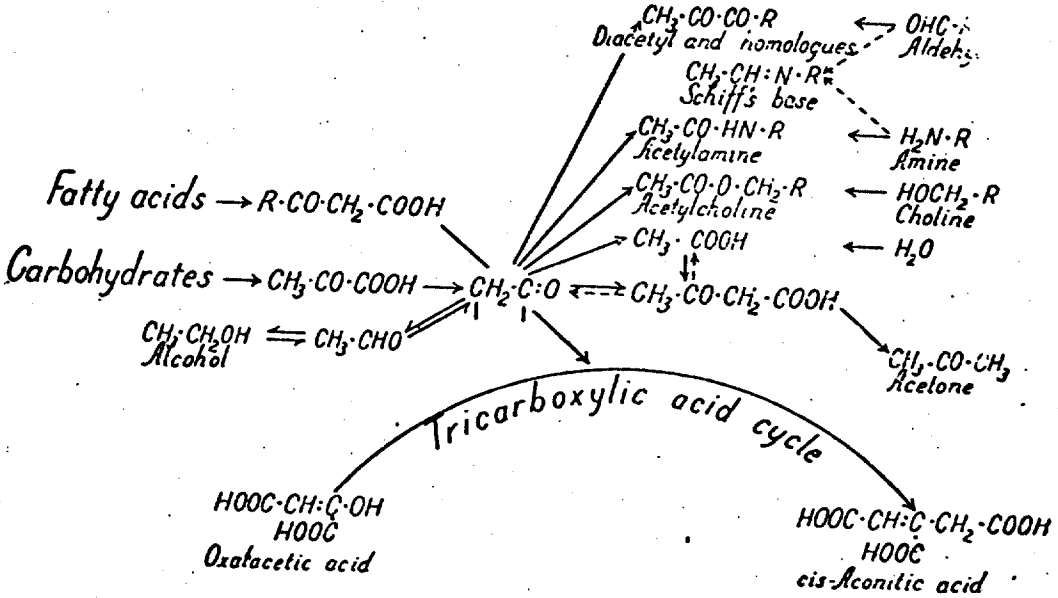


Acetylcholine



Acetylthiamine

As a summary of the foregoing we present the following scheme:



The ketene radical — formed, at least from the carbohydrate derivatives, through *Myrbäck*'s diphosphothiamine-SS redox-system — would thus be linked with the acceptors proposed above, which again would function »wie die Abfangsubstanzen von *Neuberg*» as expressed so aptly by *Reinwein* (67) with regard to arylamines. The reactions discussed above would thus indeed be regarded merely as competitive branches of metabolic acetylation. Our experimental results also seem to support strongly this view.

As *Martius* suggest, and as we have earlier stressed (78), the formula $CH_2 = C : O$ is intended less to describe the scarcely ever discernible structure of this short-lived radical than its ketene-like mode of reaction. What will be its relation to *Lipmann*'s acetylphosphate, $CH_3 \cdot CO \cdot O \cdot PO_3H_2$, seems still unsettled in spite of the facts that some workers (15, 56) have wanted to see in acetylphosphate the acetylating component, and that *Lipmann* (52, 53), quite recently, has shown adenosine triphosphate functioning as an energy donor in anaerobic acetylations. However, the above model reactions (pag. 311) already show that this need not be the case. And, although *Lardy* and *Ziegler*'s investigations with radioactive phosphorus (19), in which they established the reversibility of the reaction phosphopyruvic acid + ADP ⟶ pyruvic acid + ATP (cf. 29), seems now to have removed the weightiest objection against acetylphosphate, it should be observed that, according to *Hallman* and *Simola* (23), the effect of phosphopyruvic acid in the citric acid formation »was definitely less than that of pyruvic acid alone» (cf. also 51). Thus, »there is no experimental evidence that the reaction $CH_3COO(PO_3H_2)$ + oxalacetate ⟶ phosphorylated tricarboxylic acid occurs» (80; cf. also 26 a, 50).

Summary.

The metabolic acetylation of arylamines in animal organism was investigated with rabbits using sulphapyridine as the substrate.

Acetylation *in vivo* was found to increase almost 3-fold when vitamin B_1 was injected into animals which had been on a one-sided laboratory diet.

In vitro-experiments showed that addition of malic acid — a precursor of the tricarboxylic acid cycle — decreased acetylation while, inversely, addition of sulphapyridine decreased the formation of citric acid. — The inhibiting effect of aldehydes on acetylation seems to be ascribable to their ability to react with arylamines forming *Schiff*'s bases.

Substances able to function as hydrogen acceptors (pyruvic acid, acetaldehyde) acetylate *in vitro* relatively more strongly in anaerobic experiments, than in aerobic ones.

These results support the view that acetylation of arylamines in organism should be regarded merely as one branch of the metabolic acetylation — the others being the formations of tricarboxylic acids and of aroma substances. They also are in agreement with the assumption

that the function of *Myrbäck*'s diphosphothiamine-SS redox-system is a prerequisite to the formation of the acetylating component.

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TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
MAGNESIUM STEARATE NAS C116	17 CONF FROST(R)	*	0-5 MO.	**********	.604040	**********
			6-11 MO.	.604040	1.209080	.611200
			12-23 MO.	1.208080	4.228280	1.222580
			2-65+ YR.	1.812120	4.832320	1.833870
MAGNESIUM STEARATE NAS C116	31 CHEWING GUM(R)	*	0-5 MO.	**********	**********	**********
			6-11 MO.	**********	**********	**********
			12-23 MO.	**********	**********	**********
			2-65+ YR.	**********	**********	**********
MAGNESIUM STEARATE NAS C116	40 SOAS FLAVRS(R)	*	0-5 MO.	**********	**********	**********
			6-11 MO.	**********	.100000	**********
			12-23 MO.	**********	.200000	**********
			2-65+ YR.	.100000	.500000	.100000
MAGNESIUM STEARATE NAS C116	ALL CATEGORIES ********** ********** **********	14	0-5 MO.	1.341920	13.999140	3.111720
			6-11 MO.	15.308540	46.798870	24.888910
			12-23 MO.	24.680830	66.676670	58.738430
			2-65+ YR.	40.912140	123.209640	62.464430
MAGNESIUM SULFATE NAS C117	15 CORDM RELSH(R)	*	0-5 MO.	**********	.004000	**********
			6-11 MO.	.022000	.088000	.040000
			12-23 MO.	.112000	.304000	.140000
			2-65+ YR.	.352000	.848000	.440000
MAGNESIUM SULFATE NAS C117	23 BEV TYPE 1(R)	*	0-5 MO.	.262080	.397120	.262080
			6-11 MO.	2.478640	8.424040	2.478640
			12-23 MO.	5.918640	17.745000	5.918640
			2-65+ YR.	11.356800	30.324640	11.356800
MAGNESIUM SULFATE NAS C117	ALL CATEGORIES ********** ********** **********	*	0-5 MO.	.262080	.397120	.262080
			6-11 MO.	2.510840	8.572840	2.518640
			12-23 MO.	6.030640	18.049000	6.058640
			2-65+ YR.	11.708800	31.172940	11.796800
MALIC ACID NAS C118	01 BAKED GOODS(R)	7	0-5 MO.	.338980	.448650	.405620
			6-11 MO.	2.532300	5.164460	3.030220
			12-23 MO.	5.433650	8.953060	6.501850
			2-65+ YR.	13.678040	20.318060	16.367260
MALIC ACID NAS C118	02 BREAK CERLS(R)	4	0-5 MO.	.724920	2.053940	.861300
			6-11 MO.	26.542960	72.250360	32.011650
			12-23 MO.	31.534020	61.497380	37.486550
			2-65+ YR.	24.164000	62.504760	28.710000

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	********** POSSIBLE DAILY INTAKE, MG. ********** (AGE)	AVERAGE	HIGH A	HIGH B
MALIC ACID NAS 0118	04 FATS OILS(R)	*	0-5 MO.	.500000	.500000	2.500000
			6-11 MO.	2.800000	7.500000	14.000000
			12-23 MO.	6.300000	12.000000	31.500000
			2-65+ YR.	17.500000	31.000000	87.500000
MALIC ACID NAS 0118	05 MILK PRODS(R)	*	0-5 MO.	.508040	.481600	.958040
			6-11 MO.	6.799360	32.600890	6.799360
			12-23 MO.	5.935050	18.992160	5.935050
			2-65+ YR.	4.301550	13.123840	4.301550
MALIC ACID NAS 0118	07 FRCZN DAIRY(R)	4	0-5 MO.	.117000	.479700	.239000
			6-11 MO.	1.111500	3.083800	2.279050
			12-23 MO.	1.804800	3.954600	3.454560
			2-65+ YR.	2.995200	7.218900	6.141440
MALIC ACID NAS 0118	08 PROCSD FRUT(R)	12	0-5 MO.	26.415140	27.994120	31.218990
			6-11 MO.	114.799160	285.800000	288.754120
			12-23 MO.	222.849720	442.579140	454.044040
			2-65+ YR.	262.176460	555.379720	533.935220
MALIC ACID NAS 0118	10 MEAT PRODS(R)	4	0-5 MO.	.561220	1.479580	.920760
			6-11 MO.	10.561140	20.469160	17.317670
			12-23 MO.	15.408040	28.479300	25.288520
			2-65+ YR.	35.609480	66.277070	65.809440
MALIC ACID NAS 0118	14 PROCSD VEGS(R)	*	0-5 MO.	.000700	.002100	.001220
			6-11 MO.	.012000	.020000	.031200
			12-23 MO.	.019500	.032500	.050700
			2-65+ YR.	.042500	.071600	.110500
MALIC ACID NAS 0118	16 SOFT CANDY(R)	7	0-5 MO.	5.008640	50.088400	5.008760
			6-11 MO.	55.099040	170.299760	55.099860
			12-23 MO.	87.651200	232.001760	87.653300
			2-65+ YR.	145.250360	440.700320	145.254040
MALIC ACID NAS 0118	17 CONF FROST(R)	*	0-5 MO.	**********	.170000	**********
			6-11 MO.	.170000	.340000	**********
			12-23 MO.	.340000	1.190000	**********
			2-65+ YR.	.510000	1.360000	**********
MALIC ACID NAS 0118	18 JAM JELLY(R)	.8	0-5 MO.	**********	1.126500	**********
			6-11 MO.	25.100510	63.748340	30.727030
			12-23 MO.	11.265900	42.059360	17.702700
			2-65+ YR.	21.405210	66.468810	33.806130

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE - - - SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
MALIC ACID NAS 0118	20 GELATIN PUD(R)	5	0-5 MO.	.987200	1.232720	2.107600
			6-11 MO.	6.318080	19.151680	19.868640
			12-23 MO.	6.811560	16.584960	21.442440
			2-65+ YR.	10.065440	25.914000	31.697520
MALIC ACID NAS 0118	21 SOUPS(R)	*	0-5 MO.	.044790	.335950	.193640
			6-11 MO.	5.214870	15.277580	22.555040
			12-23 MO.	7.791720	21.516790	32.698360
			2-65+ YR.	7.097630	18.919550	30.691940
MALIC ACID NAS 0118	22 SNACK FOODS(R)	*	0-5 MO.	**********	.011900	**********
			6-11 MO.	.047600	.166600	.201320
			12-23 MO.	.130900	.369900	.553630
			2-65+ YR.	.154700	.440300	.654290
MALIC ACID NAS 0118	23 BEV TYPE I(R)	28	0-5 MO.	2.423760	3.635640	5.600240
			6-11 MO.	22.924730	78.469350	81.677270
			12-23 MO.	54.736560	164.109750	121.039420
			2-65+ YR.	105.029600	280.449730	224.010400
MALIC ACID NAS 0118	24 BEV TYPE II(R)	*	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	**********	.161940	**********
			12-23 MO.	**********	.323880	**********
			2-65+ YR.	52.630900	152.831560	62.084750
MALIC ACID NAS 0118	27 GRAVIES(R)	*	0-5 MO.	.075760	.227280	.001110
			6-11 MO.	1.060640	2.954640	1.139640
			12-23 MO.	2.724240	7.744520	2.919960
			2-65+ YR.	6.280080	16.156800	6.732120
MALIC ACID NAS 0118	30 HARD CANDY(R)	4	0-5 MO.	.000000	.000000	.000000
			6-11 MO.	.092960	.290900	.270040
			12-23 MO.	.296880	.850640	.851120
			2-65+ YR.	.593760	1.682320	1.662240
MALIC ACID NAS 0118	31 CHEWING GUM(R)	*	0-5 MO.	**********	**********	**********
			6-11 MO.	.735000	.735000	.735000
			12-23 MO.	.735000	2.205000	.735000
			2-65+ YR.	1.470000	2.940000	1.470000
MALIC ACID NAS 0118	34 INS COF TEA(R)	*	0-5 MO.	.050000	.825000	.600000
			6-11 MO.	1.325000	3.275000	15.900000
			12-23 MO.	1.550000	5.600000	18.600000
			2-65+ YR.	30.275000	64.850000	363.300000

TABLE 13, PART A -- POSSIBLE DAILY INTAKES OF NAS APPENDIX A SUBSTANCES (GROUPS I & II), PER FOOD CATEGORY AND TOTAL DIETARY, BASED ON FOOD CONSUMPTION BY TOTAL SAMPLE -- SEE EXPLANATORY NOTES IN EXHIBITS SECTION

SUBSTANCE NAME (SURVEY NO.)	FOOD CATEGORY NO. NAME	# OF FIRMS	(AGE)	POSSIBLE DAILY INTAKE, MG. AVERAGE	HIGH A	HIGH B
MALIC ACID NAS 0118	ALL CATEGORIES **********	74	0-5 MO. 6-11 MO. 12-23 MO. 2-65+ YR.	21.837160 283.706830 463.302000 745.632710	61.075070 810.901220 1069.961930 1829.476970	41.121290 515.866430 870.390000 1684.019550
MANGANESE CHLORIDE NAS 0119	83 FORMULAS(R)	*	0-5 MO. 6-11 MO. 12-23 MO.	165.074100 21.409200 6.880000	182.499000 102.006700 1.940000	**********
MANGANESE CHLORIDE NAS 0119	ALL CATEGORIES **********	*	0-5 MO. 6-11 MO. 12-23 MO.	165.074100 21.409200 6.880000	182.499000 102.006700 1.940000	**********
MANGANESE SULFATE NAS 0124	01 BAKED GOODS(R)	*	0-5 MO. 6-11 MO. 12-23 MO. 2-65+ YR.	.910000 6.850000 14.710000 37.044000	1.210000 13.986000 24.246000 55.026000	.910000 6.850000 14.710000 37.044000
MANGANESE SULFATE NAS 0124	05 MILK PRODS(R)	6	0-5 MO. 6-11 MO. 12-23 MO. 2-65+ YR.	.024100 .415080 .065150 .266600	.026800 2.010670 1.168480 .800020	.013100 .092000 .017750 .375200
MANGANESE SULFATE NAS 0124	10 MEAT PRODS(R)	*	0-5 MO. 6-11 MO. 12-23 MO. 2-65+ YR.	.006600 .124200 .181200 .470400	.017400 .334000 .211400 .780600	.006600 .124200 .181200 .470400
MANGANESE SULFATE NAS 0124	11 POULTRY(R)	*	0-5 MO. 6-11 MO. 12-23 MO. 2-65+ YR.	.003000 .023400 .039600 .077400	.013800 .079200 .110400 .196800	.003000 .023400 .039600 .077400
MANGANESE SULFATE NAS 0124	13 FISH PRODS(R)	*	0-5 MO. 6-11 MO. 12-23 MO. 2-65+ YR.	.000600 .007800 .032400 .074400	.001800 .029400 .081000 .185400	.000600 .007800 .032400 .074400
MANGANESE SULFATE NAS 0124	22 SNACK FOODS(R)	*	0-5 MO. 6-11 MO. 12-23 MO. 2-65+ YR.	********** .000000 .000000 .000000	.000000 .000000 .000000	********** .000000 .000000 .000000

No.	Name	Synonym	Formula	Beil. Ref.	Formula Weight
3965	**Leucyl glycine** (*dl*)	.....	$C_5H_{12}NCO{\cdot}NHCH_2{\cdot}CO_2H$	IV-448	188.23
3966	**Levulin**	.....	$C_6H_{10}O_5$	I-925	162.14
3967	**Levulinic acid**	acetopropionic acid	$CH_3CO(CH_2)_2CO_2H$	III-671	116.12
3968	aldehyde	.....	$CH_3CO(CH_2)_2CHO$	I-774	100.12
3969	**Lichenin**	moss starch	$(C_6H_{10}O_5)_x$	.....	(162.14)
3970[1]	**Lignoceric acid**	.....	$C_{24}H_{48}O_2$	II-393	368.65
3971	**Limettin**	5,7-diMeO-coumarin	$(CH_3O)_2C_9H_4O_2$	XVIII-97	206.20
3972	**Limonene** (*dl*)	dipentene	$C_{10}H_{16}$	V-137	136.24
3973	**Limonene** (*d* or *l*)	*p*-menthadiene-1,8(9)	$C_{10}H_{16}$	V-133	136.24
3974	**Linalool** (*d*)†	coriandrol	$C_{10}H_{18}O$	I-461	154.25
3975	**Linalyl acetate**	bergamol	$CH_3CO_2{\cdot}C_{10}H_{17}$	II-141	196.29
3976	**Linoleic acid**	octadecadienoic acid	$C_{18}H_{32}O_2$	II-496	280.45
3977	**Linolenic acid**	octadecatrienoic acid	$C_{18}H_{30}O_2$	II-499	278.44
3978	**Lophine**	triphenyl-imidazole	$(C_6H_5)_3C{:}C{\cdot}N{:}C{\cdot}NH$	XXIII-318	296.38
3979	**Lumisterol**	.....	$C_{28}H_{43}OH$	.....	396.66
3980	**Luteol**	oxychloro-diphenyl-quinoxaline	$C_{19}H_{14}ONCl$	.....	307.79
3981	**Lutidine** (2,5)‡	dimethyl-pyridine	$(CH_3)_2C_5H_3N$	XX-244	107.16
3982	**Lutidine** (2,4)(α,γ)	dimethyl-pyridine	$(CH_3)_2C_5H_3N$	XX-244	107.16
3983	**Lutidine** (2,6)(α,α')	dimethyl-pyridine	$(CH_3)_2C_5H_3N$	XX-244	107.16
3984	**Lutidine** (3,4)(β,γ)	dimethyl-pyridine	$(CH_3)_2C_5H_3N$	XX-246	107.16
3985	**Lycopene**	lycopin	$C_{40}H_{56}$	XXX-81	536.89
3986	**Lysine** (*dl*)	α,ϵ-diaminocaproic acid	$NH_2{\cdot}(CH_2)_4{\cdot}CHNH_2{\cdot}CO_2H$	IV-436	146.19
3987	dihydrochloride	.....	$C_6H_{14}O_2N_2{\cdot}2HCl$	IV-437	219.11
3988	**Lysine** (*l*+)	.....	$(NH_2)_2C_5H_9{\cdot}CO_2H$	IV-435	146.19
3989	hydrochloride (*d*)	.....	$C_6H_{14}O_2N_2{\cdot}2HCl$	IV-436	219.11
3990	monohydrochloride	(*dl*)	$C_6H_{14}O_2N_2{\cdot}HCl$	.....	182.65
3991	**Lyxose** (α)(*d*)	.....	$CH_2(CHOH)_4{\cdot}O$	XXXI-56	150.13
3992	**Lyxose** (β)(*d*)	.....	$CH_2(CHOH)_4{\cdot}O$	XXXI-56	150.13
3993	**Malachite green**§	benzaldehyde green; (zinc salt)	$3C_{23}H_{25}N_2Cl{\cdot}2ZnCl_2{\cdot}2H_2O$	XIII-745	1403.35
3994	green	(oxalate salt)	$2C_{23}H_{25}N_2{\cdot}C_2HO_4{\cdot}H_2C_2O_4$	XIII-745	927.03
3995	**Maleic acid** (*cis*)	butendioic acid; toxilic acid	$(:CH{\cdot}CO_2H)_2$	II-748	116.07
3996	anhydride	.....	$(:CH{\cdot}CO)_2O$	XVII-432	98.06
3997	**Malic acid** (*dl*)	hydroxysuccinic acid	$HO_2C{\cdot}CHOH{\cdot}CH_2{\cdot}CO_2H$	III-435	134.09
3998	acid (*d* or *l*)	.....	$HO{\cdot}C_2H_3(CO_2H)_2$	III-417	134.09
3999	Ca acid salt (*l*)	Ca bimalate	$Ca(HC_4H_4O_5)_2{\cdot}6H_2O$	.....	414.33
4000	acid (α), *iso*-	methyl tartronic acid	$CH_3{\cdot}C(OH)(CO_2H)_2$	III-440	134.09
4001	amide (*l*)	*l*-malamide	$HO{\cdot}C_2H_3(CO{\cdot}NH_2)_2$	III-435	132.12
4002	**Malonic acid**	propandioic acid	$CH_2{:}(CO_2H)_2$	II-566	104.06
4003	Ca salt	calcium malonate	$CaC_3H_2O_4{\cdot}4H_2O$	II-570	214.19
4004	amide	malonamide	$CH_2{:}(CO{\cdot}NH_2)_2$	II-582	102.09

† See also No. 2518.
‡ See also No. 3147.
§ In medicinal use without $ZnCl_2$

Levulose 3616
Lewisite 1473
Light green 3594
Lilacin, cf. glcds.
Linalool tetrahydride 2519
Linaloolone 2515
Linamarin, cf. glcds.
Lindol 6351
Litmopyrin 117
Lobeline, cf. alkd.
Lodal 2413
Loretin 3888
Luminal 5113
Luminol 359
Lupanine, cf. alkd.
Lupinidine, cf. alkd.
Lupinine, cf. alkd.
Lutein 6452
Luteosterone 5334
Lutidinic acid 5498

250a

Name	Function, Usage	Levels of Use
Lactic Acid	Acid	
	Used as acidulant in beverages, candy	
	Olives, used in brine as acidulant and preservative	0.2%
	Brewing industry. Up to	0.05%
	Dried egg whites	
	Cottage cheese	
	Confections	2.0%
	Bread, rolls, buns, etc. In such quantity that the pH of the finished bread is not less than 4.5	
	Cheese products. Same as for Acetic Acid	
	Frozen desserts, sherbets, and ices	
	Fruit jelly, butter, preserves, jams. Sufficient amount may be added to compensate for deficiency of fruit acidity	
Magnesium Carbonate	Alkali	
	Neutralizer for sour-cream butter, ice cream	
	Cacao products. Same as for Ammonium Carbonate	
	Mixed with benzoyl peroxide for bleaching flour	
	Mixed with benzoyl peroxide for bleaching milk for certain cheeses	
	Canned peas. May be used in such quantity that the pH does not exceed 8.0	
Magnesium Hydroxide	Alkali	
	Canned peas. In such quantity that the pH does not exceed 8.0	
Magnesium Oxide	Alkali	
	Used as neutralizer in frozen dairy products, butter	
	Cacao products. Same as for Ammonium Carbonate	
	Canned peas. May be used in such quantity that the pH does not exceed 8.0	
* Malic Acid	Acid	
	Frozen dairy products, beverages, bakery products, etc.	
	Confectionery. Up to	4.0%
	Fruit butter, jelly, jams, preserves. Sufficient amount may be added to compensate for deficiency of fruit acidity. Artificially sweetened preserves	
✓ Phosphate, Calcium, monobasic (Calcium acid phosphate)	Buffer	
	Prepared mixes (pancake, muffin, cake, biscuit)	1.0 - 1.5%
	Leavening ingredient for various crackers and cookies	0.05 - 0.20%
	Pancake flour	2.5%
	Self-rising flours, self-rising white and yellow corn-meals. Same as for Carbonate, Sodium bi-	
	Phosphated flour	0.25 - 0.75%
	Artificially sweetened jelly and preserves	
	Canned potatoes, canned green or red sweet peppers	
	Canned tomatoes	
Phosphate, Calcium, tribasic	Buffer	
	Used for pH adjustment in frozen dairy products	0.0016 - 0.005%
	Beverages	0.09 - 0.1%
	Confectionery	0.0025 - 0.006%
	Baked goods	0.004 - 0.03%
Phosphate, Sodium, dibasic (Disodium phosphate)	Buffer	
	Used to adjust acidity of various foods	
	Chocolate products	0.4 - 0.8%

Calcium phosphates

l-MALIC ACID

Chemical formula: $HOOCCHOHCH_2COOH$

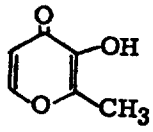
Flavors in which used:
Fruit, maple

Natural food occurrence:
Coffee, peaches, rhubarb root, vanilla

Foods in which used:	Approx. Avg Maximum ppm
Beverages	380
Ice cream, ices	390
Candy	420
Baked goods	0.60, 1.5

MALTOL
3-Hydroxy-2-methyl-γ-pyrone

Chemical formula:



Flavors in which used:
Chocolate, coffee, fruit, maple, nut, vanilla

Natural food occurrence:
Chicory, roasted malt

Foods in which used:	Approx. Avg Maximum ppm
Beverages	3.1
Ice cream, ices	8.7
Candy	31
Baked goods	30
Gelatin desserts	7.5
Chewing gum	90
Jelly	15

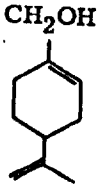
MELONAL
(See 2,6-Dimethyl-5-heptenal)

p-MENTHA-1,5-DIENE
(See α-Phellandrene)

d-p-MENTHA-1,8-DIENE
(See d-Limonene)

p-MENTHA-1,8-DIEN-7-OL
1,8-p-Menthadien-7-ol

Chemical formula:



Flavors in which used:
Citrus, fruit, mint, vanilla

Natural food occurrence:
Caraway

Foods in which used:	Approx. Avg Maximum ppm
Beverages	0.50, 1
Ice cream, ices	0.50, 1
Candy	20
Baked goods	10, 50

p-MENTHA-6,8-DIEN-2-OL
(See Carveol)

6,8(9)-p-MENTHADIEN-2-ONE
(See Carvone)

3-p-MENTHANOL
(See Menthol)

p-MENTHAN-3-ONE
(See Menthone)

p-MENTH-1,4(8)-DIENE
(See Terpinolene)

p-MENTH-1-EN-8-OL
(See α-Terpineol)

p-MENTH-8-EN-3-OL
(See Isopulegol)

1-p-MENTHEN-4-OL
(4-Carvomenthenol)

8-p-MENTHEN-2-OL
(See Dihydrocarveol)

Δ-4(8)-p-MENTHEN-3-ONE
(See Pulegone)

Δ-8(9)-p-MENTHEN-3-ONE
(See Isopulegone)

p-MENTH-1-EN-3-ONE
(See d-Piperitone)

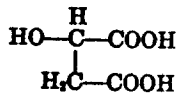
153. N. Sax, *Dangerous Properties of Industrial Materials*, 2nd ed., Reinhold Publishing Corp., New York, 1963.
154. W. Ashcroft and I. Clifford, *Chem. Prod.* 11 (1961).

B. Dmuchovsky and J. E. Franz
Monsanto Company

MALIC ACID

Malic acid (hydroxysuccinic acid, hydroxybutanedioic acid, 1-hydroxy-1,2-ethanedicarboxylic acid) is a white or nearly white powdery, granular, or crystalline material. The levorotatory isomer occurs widely distributed in the vegetable kingdom. Malic acid has not enjoyed widespread industrial application to date because of limited availability and high cost. Recent large-scale production facilities for the racemic mixture of malic acid isomers will allow complete exploration of the commercial potential of this acid.

The presence of a hydroxyl group, as well as of two carboxyl groups, results in a highly soluble molecule capable of the reactions usually attributable to these groups.



Malic acid, the principal acid found in apples, is sometimes referred to as "apple acid." The characteristic flavor of malic acid has been used advantageously in the manufacture of apple jams, jellies, candy, and beverage products.

Occurrence

Malic acid occurs widely in biological systems. It is the predominant acid in numerous fruits, where it contributes significantly to the flavor, especially in unripe apples (Table 1). It is present in grapes together with a usually lesser amount of tartaric acid. The change in flavor as some of these fruits ripen is associated with a reduction in malic acid content and a simultaneous increase in sugar. Malic acid occurs in relatively low concentrations thus making the isolation from natural sources expensive and impractical. An ion-exchange process has been published (1) for the

Table 1. Malic Acid in Fruits (3–5)

Fruit	% of total acid	Fruit	% of total acid
apple	97.2	orange pulp	trace
apricot	23.7–69.8	peach	50.0–96.2
banana	53.7–92.3	pear	33.0–86.6
blueberry	6.0	persimmon	100.0
cherry	94.2	pineapple	12.5
cranberry	19.1–23.5	plum	98.5
gooseberry	46.2	quince	100.0
grape (Concord)	60.0	rhubarb	77.0
grapefruit	5.6	strawberry	9.9–11.0
lemon	4.5	watermelon	100.0
orange peel	59.6–80.0		

isolation of this acid from apple juice which may contain from 0.4 to 0.7% malic acid (2). It can also be obtained from its calcium salt in "sugar sand," a by-product of the maple sugar industry.

In addition to its presence in sour fruits, malic acid has been found in cultures of a variety of microorganisms including *Aspergilli* (6), yeast (7), *Sclerotinias* (8), and *Penicillium brevi-compactum* (9). Among the *Rhizopi*, it occurs together with L(+)-lactic acid and fumaric acid. Yields of levorotatory malic acid as high as 55 g/100 g of a D-glucose, amounting to 74% of theory, have been reported for *Aspergillus flavus* and *Aspergillus parasiticus* (6). Iron, manganese, chromium, or aluminum ions reportedly enhance malic acid production.

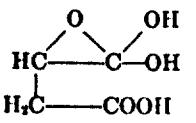
Malic acid is of interest to the biochemist because it is a participant in two respiratory metabolic cycles—the Krebs tricarboxylic acid cycle and the glyoxylic acid cycle. These metabolic cycles appear to play two essential roles in cellular metabolism. They account for the terminal oxidation system which supplies energy and provide the carbon skeletons from which many of the amino acids of proteins are derived.

Chemical and Physical Properties

Malic acid crystallizes from aqueous solutions as white, translucent anhydrous crystals which melt at 129°C and decompose at about 180°C to yield fumaric and maleic acids. Under normal conditions, malic acid is stable; however, the pure crystals are somewhat hygroscopic and under conditions of high humidity tend to liquefy. A 50.4% weight gain was observed after six days at 98% relative humidity at 25°C (10).

Malic acid is a strong organic acid. The first dissociation constant is reported to be 4.0×10^{-4} and the second dissociation constant is 9×10^{-6} at 25°C. The pH of a 0.1% solution is 2.8, and of a 1.0% solution is 2.4. It is soluble in water to the extent of 48 g at 5°C, 58 g at 25°C, and 80 g at 75°C in 100 g. Many physical properties are very similar to those of citric acid (11). The chelation index of malic acid is 46.3 compared with 70.5 for citric acid, 52.1 for tartaric acid, 13.5 for lactic acid, and 4.3 for acetic acid (12).

Because of the presence of an asymmetric carbon atom, malic acid is optically active. Much study has been devoted to the effect of dilution on the optical activity of malic acid (13). The naturally occurring acid shows a most peculiar behavior in this respect; a 34% solution at 20°C is optically inactive. Dilution results in an increasing levo specific rotation, while more concentrated solutions show an increasing dextro rotation. The peculiar behavior observed in the optical activity of malic acid led Bancroft and Davis (13) to conclude that an additional form, the epoxide, occurs in solutions. Some of the apparent anomalies are explained by the fact that the direction of rotation of the linear and epoxide forms is reversed.

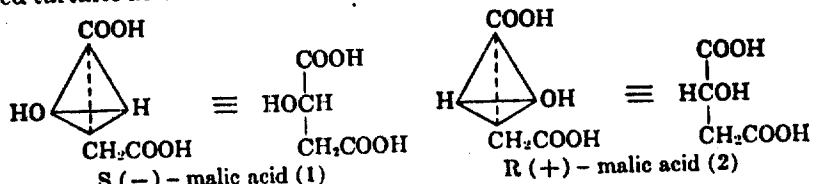


epoxide form of malic acid

Synthetic malic acid, a racemic mixture, is best resolved by crystallization of its cinchonine salts. When partly racemized malic acid is crystallized as the ammonium molybdate derivative, the form present in excess crystallizes first.

Configuration

Fischer (14) concluded that the naturally occurring levorotatory isomer has the spatial configuration (1) as a result of the work carried out by Bremer (15), in which he reduced tartaric acid to malic acid.

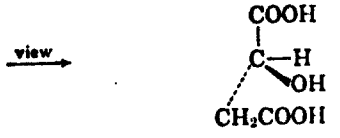


S (−) – malic acid (1) R (+) – malic acid (2)

The other isomer (2) which exists as a result of the asymmetric carbon atom is assigned the *R*-configuration symbol.

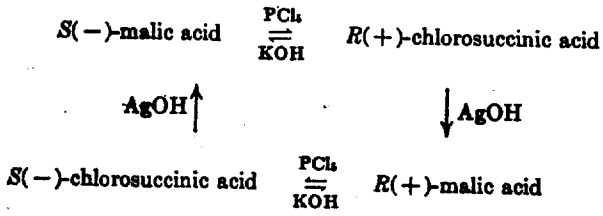
The configuration of a molecule can be absolutely designated by the use of a three-dimensional drawing as in (1) or by use of the Fischer projection indicated in (2); however, it is frequently desirable to have names which designate configuration. Conventions have been adopted generally by chemists based upon correlation with glyceraldehyde using the symbols D,L or by correlation with the amino acid serine using the designations D,L. These systems conflict when naming certain molecules with asymmetric carbons, with malic acid and tartaric acid as examples. Frequently, authors have not indicated the system they were using by proper subscripts. Often the lower-case letters *d* and *l* used to designate rotation were interpreted as configuration designations. Much confusion in the literature understandably exists. Neither system has been internationally accepted, particularly because each is unsuitable for molecules containing more than one asymmetric carbon atom.

A system proposed by Cahn and Ingold (16) provided a means to designate unequivocally the absolute configuration of each asymmetric carbon atom in a molecule. This procedure, known as the "sequence rule," or the "*R* and *S* system," has rapidly gained acceptance in the current literature. The proper designation, *R* or *S*, is determined by assigning a priority to each group attached to the asymmetric atom. The priority of each group is based upon the atomic number of the atom in the group linked directly to the asymmetric atom. Thus, oxygen has a higher priority than carbon. If two or more atoms directly linked are the same, the priority is then assigned on the basis of the atomic numbers of the atoms attached to the atom directly linked to the asymmetric atom. If the attached atoms are the same, the priority is based upon the next atoms attached along the chain until the priority is determined. Thus, the *n*-propyl group has a higher priority than the ethyl group. Atoms connected by double bonds are treated as if two single bonds existed, thus a carbonyl group —C=O is treated as if it were O—C—O. The following atoms and groups listed as examples are in order of decreasing priority: I, Br, Ca, Cl, S, Na, F, O, N, —$COOCH_3$, —COOH, —$CONH_2$, —C, *N*-C_6H_5—, —$C(CH_3)_3$, —$CH(CH_3)_2$, —CH_2CH_3, —CH_3, T, D, H. Note that isotopes with the highest mass number are given highest priority. A three-dimensional drawing of the asymmetric carbon and its attached groups is drawn and viewed from the side remote from the group of lowest priority.



The order of decreasing priority of the three groups constituting the triangle nearest the observer is determined. If the priority is observed to decrease clockwise the configuration notation *R* is assigned (Latin *rectus*, right). A priority sequence which decreases counterclockwise is designated *S* (Latin *sinister*, left). Each asymmetric atom can be designated in turn. It should be noted that pseudo-asymmetric carbons are designated by assigning *R* a higher priority than *S*. Naturally occurring malic acid is thus designated *S*(−)-malic acid, where (−) indicates the substance is levorotatory. Dextrorotatory tartaric acid is designated *R,R*(+)-tartaric acid. During the development of the sequence rule, some of the earlier procedures were amended. Cahn (17) has described the now-accepted procedures in more detail than this introduction to the system.

When the following reaction scheme was carried out, one of the most remarkable phenomena of organic chemistry, the Walden inversion, was discovered (18).

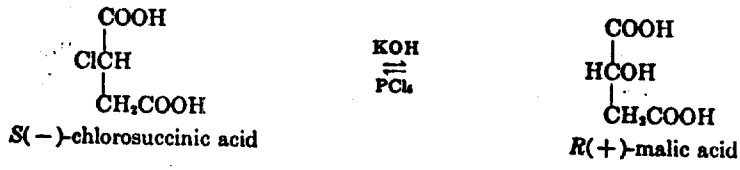


This work indicated to early investigators that the formation of a compound is not as simple as it appears. Realization that the entering group does not occupy the same position on the asymmetric carbon as that occupied by the group which is removed gave evidence to the need for a mechanistic approach to organic chemistry.

The Walden inversion may be envisioned as a concerted reaction whereby a nucleophilic reagent collides with the compound undergoing reaction, with the collision occurring at the rear of the carbon atom from which the leaving group departs. In modern mechanistic terms this is described as an S_N2 reaction because it is a bimolecular nucleophilic substitution.

Studies show that *S*(−)-malic acid and *S*(−)-chlorosuccinic acid have the same configuration, as shown below.

The inversion occurs during reaction with potassium hydroxide or phosphorus pentachloride.



The correct name for the first substance would be *S*(−)-chlorosuccinic acid, the *S* showing its absolute configuration. In the following reactions, *R* and *S* will be used in reference to configuration of the structures.

Other reaction schemes were investigated with sometimes surprising results—until the following mechanistic approach was used to explain the facts (18):

$$R(+)\text{-chlorosuccinic acid} \xrightleftharpoons[PCl_5]{KOH} S(-)\text{-malic acid}$$

$$AgOH \downarrow\uparrow SOCl_2, \text{ pyridine} \qquad AgOH \uparrow\downarrow SOCl_2, \text{ pyridine}$$

$$R(+)\text{-malic acid} \xrightleftharpoons[PCl_5]{KOH} S(-)\text{-chlorosuccinic acid}$$

In the foregoing series of reactions, $SOCl_2$ and AgOH cause no inversion while KOH and PCl_5 cause inversion; however, different results are obtained with the same reagents in other solvents, especially $SOCl_2$ and ether. It was formerly thought that the inversion took place only when the asymmetric carbon was in a position alpha to a carboxyl group. The changes observed in the Walden inversion are of fundamental importance in organic chemistry as they are involved in all replacement reactions. Similar changes undoubtedly take place in optically inactive compounds, but there are presently no ways of detecting these changes in such structures.

Reactions

Esterification. Malic acid yields the usual diesters with an alcohol in the presence of an esterification catalyst (19–22).

Monoesters may be easily prepared by refluxing malic acid with an alcohol with boron trifluoride as a catalyst (23).

With polyhydric alcohols and polycarboxylic aromatic acids, resinous products result, yielding alkyd polyester resins (24).

Complete esterification of the molecule can be accomplished by reacting a diester of malic acid with an acid chloride, such as acetyl or stearoyl chloride (25).

Etherification. Alkyl halides in the presence of silver oxide react with alkyl malates to yield alkoxy derivatives of succinic acid, for example, α-ethoxy succinic acid, $HOOCCH_2CHOC_2H_5COOH$ (26,27).

Another and perhaps more realistic synthetic approach to ethers of malic acid is the reaction of maleic esters with sodium alkoxides resulting in the α-alkoxysuccinic esters (28).

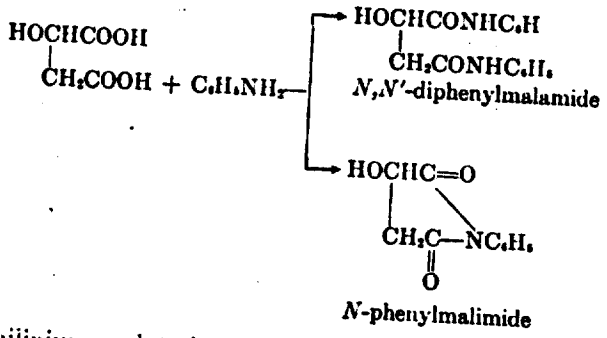
Amidation. The expected amides are obtained when alkyl esters of malic acid are treated with ammonia in alcoholic solution (29,30).

$$\begin{matrix} HOCHCOOCH_3 \\ | \\ CH_2COOCH_3 \end{matrix} \xrightarrow{2\,NH_3} \begin{matrix} HOCHCONH_2 \\ | \\ CH_2CONH_2 \end{matrix} + 2\,CH_3OH$$

Hydrazine reacts in a similar manner to yield the malic dihydrazide (31).

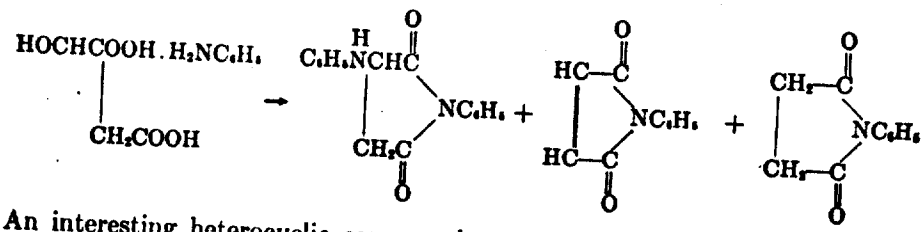
$$\begin{matrix} HOCHCOOCH_3 \\ | \\ CH_2COOCH_3 \end{matrix} + 2\,NH_2{-}NH_2 \rightarrow \begin{matrix} HOCHCONHNH_2 \\ | \\ CH_2CONHNH_2 \end{matrix}$$

Depending on the proportions of water present in the reaction, aniline and malic acid form *N,N'*-diphenylmalamide or the cyclic compound *N*-phenylmalimide (32).

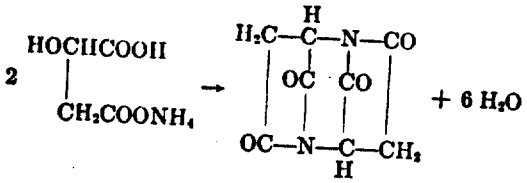


N-phenylmalimide

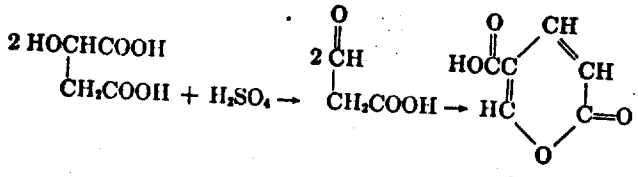
When monoanilinium malate is distilled under reduced pressure, a mixture of *C*-anilino-*N*-phenylsuccinimide, *N*-phenylmalanil, and *N*-phenylsuccinimide is obtained (33,34).



An interesting heterocyclic compound, 2,5-dioxopiperazinediacetic dilactam, results when monoammonium malate is heated (35):



Oxidation. Malic acid yields 75–80% of coumalic acid when treated with fuming sulfuric acid (36).

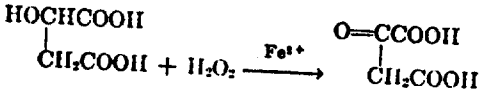


coumalic acid

Similar treatment of malic acid in the presence of phenol and substituted phenols affords a facile method of synthesizing coumarins substituted in the aromatic nucleus (37–40).

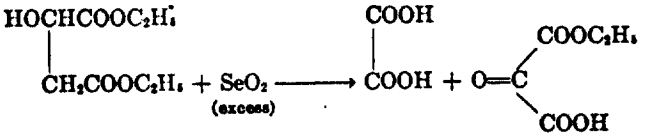
Similar reactions take place with thiophenol and substituted thiophenols, yielding interesting compounds, one of which is a red dye (41). Malic acid and 2-thiobenzoic acid, 2-HSC_6H_4COOH, in this reaction yields 3-hydroxy-1-thionaphthalic-2-aldehyde.

Oxidation of an aqueous solution of malic acid with hydrogen peroxide catalyzed by ferrous ions yields oxalacetic acid (42):

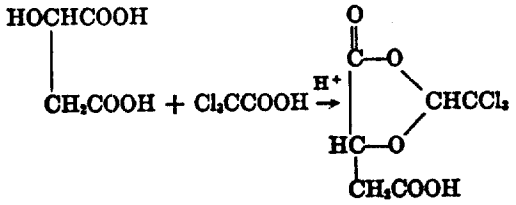


If the above oxidation is performed in the presence of chromium, ferric, or titanium ions or mixtures of these, the product is tartaric acid (43).

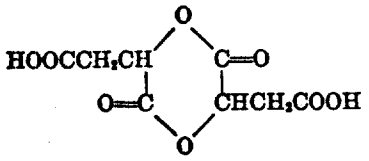
Selenium dioxide reacts with an excess of ethyl malate to yield ethyl dioxosuccinate and some ethyl fumarate (44). With an excess of oxidizing agent, ethyl malate is converted to oxalic acid and monoethyl mesoxalate (44) as follows:



Reaction with Aldehydes. Chlorals react with malic acid in the presence of sulfuric acid or other acidic catalysts to produce an interesting class of heterocyclic compounds, the 4-ketodioxolones (45,46).



Other Reactions. Malic acid does not form an anhydride but does yield two types of condensation products, linear malomalic acids and the cyclic dilactone or malide, which behaves to some degree like an anhydride (12).



malide

Manufacture

Biosyntheses. An interesting method of preparing *S*(−)-malic acid, the natural form, is based on the use of the enzyme fumarase which catalyzes the "transcrystallization" reaction (47).

$$\text{fumarate} + H_2O \xrightarrow{\text{fumarase}} \text{malate}$$

When supplied as the calcium salt, the relatively greater solubility of calcium fumarate than that of calcium malate forces the reaction to completion. Cells of one of the *Lactobacillus* species are a source for the enzyme. With *L. brevis*, a conversion of fumarate to malate has occurred with an efficiency of 99% in 24 hr. When the reaction was conducted with two-day-old liquid cultures of *L. brevis*, the reaction efficiency was 88%. Since fumaric acid is readily obtained by oxidation of benzene, "transcrystallization" might offer an economical synthesis of malic acid. "Transcrystallization" would offer the advantage over hydration of fumaric acid by heat or chemical means in that it would produce only a single optically active isomer, the naturally occurring form. Extensive studies have been made on the kinetics of the enzyme-catalyzed reversible hydration of fumarate to *S*-malate (48–50). Olefinic

hydration catalyzed by enzymes features high rates, stereospecificity, and neutral conditions.

Chemical Syntheses. *R,S*-Malic acid has been made available from a synthetic process which involves hydration of maleic or fumaric acid at elevated temperatures and pressures in the presence of either sodium hydroxide (51), H_2SO_4 (in lead-lined apparatus) (52), or various metallic (53) catalysts or without the aid of any additives (54,55).

Acidic, basic, and neutral conditions have been investigated for the nonenzymatic hydration of fumaric acid to malic acid. Most investigators have reported the preparation of malic acid from fumaric, but it is understood that maleic acid may be substituted for fumaric acid.

The kinetics and mechanisms of the nonenzymatic hydration of fumaric acid monoanion under neutral conditions have also been investigated (56).

The base-catalyzed hydration of fumarate to *R,S*-malate in water in the temperature range of 90–175° C follows a reversible first-order rate in fumarate and malate. Rates are proportional to base (NaOH) concentration at constant ionic strength. The racemization rate of *S*-malate catalyzed by NaOH is equal to the rate of dehydration of *R,S*-malate (57).

Equilibrium constants and rate constants for the hydration of fumaric acid to malic acid have been determined at hydrochloric acid concentrations of 1 to 4*M* in the temperature range of 125–200°C (58). The values of ΔS of hydration and dehydration support the hypothesis that the rate-determining step in the hydration reaction is a bimolecular reaction with water (58).

Reznikov and Grud'ev (52) describe the preparation of *R,S*-malic acid in a Russian patent in some detail. In essence, a 40% stock solution of maleic acid is charged into a lead-lined autoclave and hydrated under pressure to malic acid.

At room temperature maleic acid is corrosive to most metals, and at the reaction temperature of 165–170°C, maleic acid is very highly corrosive, attacking such materials as Cr–Ni steels, enamels, glass linings, and other coating structures. The Russian investigators found that the most suitable material of construction for the hydration autoclaves was lead. The lead liner is pretreated with a 4% solution of sulfuric acid to form a film of lead sulfate. This coating protects the lead liner from being attacked by maleic acid and the acid products of the reaction.

A mixture of maleic acid and 4% sulfuric acid was charged into an autoclave, with the sulfuric acid acting as the hydration catalyst. The conversion to malic acid proceeded in 65–70% yields, while 30–35% of the maleic acid was converted to fumaric acid. Since fumaric acid is not very water-soluble, upon cooling the reaction mixture, it was easily removed by filtration. The filtrate containing crude malic acid, sulfuric acid, small amounts of fumaric acid, and colored iron impurities was neutralized with $CaCO_3$ or calcium malate. The iron was converted to Fe_2O_3 with H_2O_2 and then precipitated as Prussian blue by adding sodium cyanide. This mixture was decolorized with carbon, the sludge removed by suction filtration, and then the filtrate was collected and concentrated under reduced pressure to about 20% malic acid concentration. At this stage the solution was cooled again and filtered to remove traces of calcium sulfate and residual fumaric acid. The filtrate was again concentrated under vacuum to a specific gravity of 1.34 or 80% malic acid concentration. Cooling caused crystallization of malic acid which was centrifuged, dried, sieved, and packaged.

The first mother liquor was concentrated to yield a second crop of crystals, while

the second mother liquor was converted to calcium malate which was used to neutralize the sulfuric acid in subsequent autoclavings. The fumaric acid isolated from the autoclave reaction was purified by being treated with carbon, recrystallized, and recycled in the preparation of malic acid.

A Japanese patent (59), describing a manufacturing procedure for malic acid, claims the direct hydration of maleic acid at 180°C and 150–175 lb pressure. These workers suggest that in the hydration of maleic acid fumaric acid is formed as a by-product which is slowly hydrated under the conditions of reaction. They further suggest that if an amount of fumaric acid equivalent to that formed at equilibrium conditions is charged together with the maleic acid, it is possible to hydrate maleic acid to malic in a much shorter reaction time without the formation of any additional fumaric acid.

As an example, they cite the following experiments performed at a temperature of 180°C, a pressure of 150 lb, and a reaction time of 10 hr:

Example 1

Charged to autoclave:

maleic acid	50 g
water	75 ml

Yield: 36.3 g malic acid and 17.3 g fumaric acid

Example 2

Charged to autoclave:

maleic acid	32.7 g
fumaric acid	17.3 g
water	75 ml

Yield: 36.2 g malic acid and 17.5 g fumaric acid

Example 3

Charged to autoclave:

maleic acid	32.5 g
fumaric acid	17.5 g
water	75 ml

Yield: 36.4 g malic acid and 17.5 g fumaric acid

Table 2 shows the comparison of yields and reaction times obtained when maleic acid was used alone and when 45–55% of fumaric acid based on the maleic acid was simultaneously charged to the autoclave; no catalyst was employed. The reaction was found to be independent of pressure. This concept of the Japanese workers is therefore substantiated by the increase in yield of malic acid and reduction in reaction time achieved by the addition of fumaric acid.

From the data (Table 2) these investigators postulated that both the isomerization and the hydration are equilibrium reactions.

Table 2. Comparison of Yields of Malic Acids

	Yield of malic acid at reaction times, %		
	4 hr	10 hr	26 hr
maleic–fumaric acid mixture	83	100	
maleic acid	56	62	67

Economic Data

Actual production figures for the U.S. and the world are not available. The demand in 1963 was estimated at 1 million lb (60). In 1965, the sole U.S. producer completed a plant in West Virginia with capacity rated at 20 million lb (61). This plant utilizes a continuous process (62) for which patents have been applied for (63). It is believed the process is based upon the hydration of maleic acid produced by the catalytic oxidation of benzene.

The price for technical grade malic acid has ranged between 45 and 54¢/lb for the period from about 1930–1965. The price for food grade was quoted at 62¢/lb prior to October 1965, when the price was reduced to 29.5¢/lb (64).

A new production plant with a first-stage sales target of 1000 tons/year began early in 1965 at Widnes, England. The selling price was fixed at £190 per ton, which corresponds to about 26.5¢/lb (12). Two plants known to be operating in Japan are offering malic acid at competitive prices throughout the world.

Specifications

Until a few years ago no standard specifications have existed for malic acid, with each manufacturer making his own specifications. Recently, however, specifications were established for a *Food Chemicals Codex* (FCC) grade (65) (see Table 3).

Table 3. Specifications for Malic Acid FCC (65)

appearance, white or nearly white powder or granules
solubility, 1.0 g dissolves in 0.8 ml of water
1.0 g dissolves in 1.4 ml of ethanol
assay, 99.5% by titration
melting point, 128–129°C
arsenic, $\leq$3 ppm
fumaric acid, $\leq$0.5%
heavy metals, $\leq$20 ppm as lead
maleic acid, $\leq$0.05%
residue on ignition, $\leq$0.1%
water-insoluble matter, $\leq$0.1%

Food Chemicals Codex is being prepared by the National Academy of Sciences–National Research Council from a research grant from the U.S. Public Health Service and grants from a large segment of U.S. industry. These industries have played an important role in developing and reviewing these specifications which are now being utilized by government as well as private agencies. Quality of malic acid has varied widely from manufacturer to manufacturer and even from lot to lot from the same source. The Codex specifications will result in a much more uniform product, particularly since the presence of fumaric acid and maleic acid as impurities is inherent in some chemical syntheses.

Standard Test Methods

The FCC procedure for determining maleic and fumaric acids in malic acid is based on a polarographic method. A gas chromatographic procedure based upon the trimethyl silyl derivatives has been developed (66) but it has not been published nor is it yet an official *Food Chemicals Codex* method.

Uses

Usage has been limited to date; however, it is expected that the market will expand as a result of greater availability.

The U.S. Food and Drug Administration has declared malic acid to be "GRAS" (generally recognized as safe) (67). Only the levorotatory malic acid isomer has approval in the U.S. for use as a "synthetic flavor" (68).

Extensive evaluations have been conducted in the food industry with malic acid in a wide variety of formulations. Applications to date have been restricted to specialty items. Malic acid reportedly enhances the flavor of fruit products, such as those from apples, peaches, and cherries (61).

Malic acid is the acidulant employed in a new peach drink being offered by a packer in Georgia (69). A confectionery product which consists of dextrose, malic acid, and a fruit flavor in tablet form is being promoted. One malic acid manufacturer promotes his product by distributing apple-flavored hard candy to potential customers. Another manufacturer using malic acid in some of his ice-cream fountain syrups claims better flavor. A pharmaceutical manufacturer in the United Kingdom is offering "teeth-cleaning tablets" which contain malic acid (12).

The use of citric acid to protect the pectin during its recovery from orange peels is known (70). Since malic acid is the major acid found in orange peels and citric predominates in the pulp (71), malic acid may find use in this application.

The relationship of malic acid to the flavor of tobacco and its incorporation into tobacco products to improve flavor are described in a patent (72). Malic acid derivatives are claimed to impart enhanced flavor (73). Polyester and alkyd resins formed from malic acid are used to a limited degree as plastics (74). The addition of malic acid to a shellac or varnish paint containing $MgSiO_3$ is claimed to prevent skinning (75). The esters of malic acid are reported to prevent spattering of oleomargarine and other cooking fats (76).

Sodium malate is reported to be valuable in hepatic malfunctioning, especially in hyperammoniemia. Sodium malate is also reported useful as a condiment in salt-free diets since it has a flavor much like that of sodium chloride (12).

Calcium malate can be used as a stabilizer for calcium lactate injections (77).

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MALONIC ACID AND DERIVATIVES

Malonic acid (propanedioic acid (IUPAC), methanedicarboxylic acid), HOOC-CH_2COOH, was discovered by Dessaignes, who isolated it as a product of the oxidation of malic acid, $HOOCCHOHCH_2COOH$, and for this reason named it malonic acid. Von Lippmann found the calcium salt of the acid as a constituent of beet juice. The first syntheses were carried out by Kolbe and Müller, essentially in the same way as the acid is prepared today. The literature on malonic acid and its derivatives is so voluminous that only a brief survey of its chemistry can be given here. Malonic acid derivatives are used in the preparation of barbituric acid, vitamins B_1 and B_6, amino acids, and hypnotics.

Malonic acid, as a member of the class of dicarboxylic acids (see Vol. 1, p. 243), shows all the reactions of aliphatic dicarboxylic acids. In two respects, however, its behavior is fundamentally different from that of its homologs. It easily loses one carboxylic group as carbon dioxide, and the methylene group shows a pronounced reactivity. Both properties are due to the grouping —$COCH_2CO$— of β-dicarbonyl compounds which, besides malonic acid, comprise β-keto acids like acetoacetic acid and β-diketones (see p. 147).

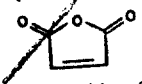
Physical and Chemical Properties

Physical Properties. Malonic acid is trimorphic; there are two triclinic forms, one stable up to 94°C and one unstable, and a monoclinic form stable above 94°C. It is very soluble in water and alcohols. In pyridine and ether the solubility is 5–10%. The alkali salts are also very soluble in water. The barium and lead salts are insoluble and can be used for the determination of the acid.

The melting and boiling points of the more important functional derivatives of malonic acid are listed in Table 1, and the boiling points of some technically important *C*-substituted malonic esters in Table 2.

300a

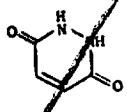
production by catalytic vapor-phase oxidation of benzene or other suitable hydrocarbons: Weiss, Downs, *Ind. Eng. Chem.* 12, 228 (1920); U.S. pat. 1,318,633 (1920 to Barrett Co.). Many other syntheses. *Review* of commercial methods of manufacture: Ashcroft, Clifford, *Chem. Prods.* 24, 11 (1961); *C.A.* 55, 9724d (1961).



Orthorhombic needles from chloroform or by sublimation. Commercial grades are furnished in fused form, as briquettes. Sublimes readily; d 1.48. mp 52.8°. bp$_{760}$ 202.0°; bp$_{400}$ 179.5°; bp$_{200}$ 165.9°; bp$_{100}$ 135.8°; bp$_{60}$ 122.0°; bp$_{40}$ 111.8°; bp$_{20}$ 95.0°; bp$_{10}$ 78.7°; bp$_{5}$ 63.4°. Specific heat: 0.285 (solid); 0.396 (liq). Soluble in water, forming maleic acid. Solubility at 25° in 100 g solvent: acetone 227 g; ethyl acetate 112 g; chloroform 52.5 g; benzene 50 g; toluene 23.4 g; *o*-xylene 19.4 g; carbon tetrachloride 0.60 g; ligroin 0.25 g. Soluble in dioxane. Soluble in alcohol with ester formation.

USE: In Diels-Alder syntheses (diene syntheses), in copolymerization reactions, manuf alkyd-type of resins, due intermediates, pharmaceuticals, agricultural chemicals (maleic hydrazide, Malathion). *Human Toxicity:* Powerful irritant. *Warning! Causes burns.* Avoid contact with skin, eyes, clothing. Inhalation can cause pulmonary edema. Avoid exposure to concd vapor. In case of contact, immediately flush skin or eyes with plenty of water for at least 15 min.

Maleic Hydrazide. *1,2-Dihydro-3,6-pyridazinedione; maleic acid hydrazide;* Malazide; MH; Regulox. $C_4H_4N_2O_2$; mol wt 112.09. C 42.86%, H 3.60%, N 24.99%, O 28.55%. Prepd by treating maleic anhydride with hydrazine hydrate in alcohol: Arndt *et al.*, *C.A.* 43, 579 (1949); *see also* Curtius, Foerstinger, *J. prakt. Chem.* (2) 51, 391 (1895). From maleic acid and a hydrazine salt of a strong inorganic acid: Harris, Schoene, U.S. pat. 2,575,954 (1951 to U.S. Rubber Co.). *Review:* Massey, *Mfg. Chemist* 26, 197-200 (1955). Alternate prepn from hydrazine sulfate and maleic anhydride in aq NaOH: Amatsu, Karasawa, *C.A.* 51, 18014c (1957); from hydrazine hydrate and maleic anhydride in glacial acetic acid: Feuer *et al.*, *J. Am. Chem. Soc.* 80, 3790 (1958).



Crystals from water, dec 260°. mp over 300° (Feuer *et al.*, *loc. cit.*). Slightly sol in hot alcohol, more sol in hot water. LD_{50} orally in rats: 4.0 g/kg.

USE: Experimentally in horticulture and agriculture. Has the ability to inhibit growth of plants without killing them: Schoene, Hoffmann, *Science* 109, 588 (1949). To control suckering of tobacco. In the synthesis of pyridazine. *Human Toxicity:* Most hydrazides are highly toxic. In exptl animals acute exposure has produced CNS disturbances and chronic exposure has produced liver damage.

Maleuric Acid. *N-Carbamoylmaleamic acid;* maleylurea. $C_5H_6N_2O_4$; mol wt 158.11. C 37.98%, H 3.83%, N 17.72%, O 40.48%. Prepd from maleic anhydride and urea: Dunlap, Phelps, *Am. Chem. J.* 19, 492 (1897); Batt *et al.*, *J. Am. Chem. Soc.* 76, 3663 (1954).

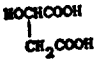
Crystals from hot water, dec 158-5°. Absorption max 235 mμ ($\epsilon \times 10^{-3} = 8.72$). Soluble in hot acetic acid. Practically insol in cold water, cold acetic acid, acetone, ligroin, chloroform, alcohol, ether.

Methyl ester, crystals, mp 113-114°. Soluble in hot water, methanol, ethanol, acetone, dioxane.

Butyl ester, crystals, mp 95-98°. Soluble in ethanol, acetone, benzene, chloroform.

Dodecyl ester, crystals, mp 110-111°. Soluble in dioxane.

Malic Acid. Hydroxysuccinic acid. $C_4H_6O_5$; mol wt 134.09. C 35.83%, H 4.51%, O 59.66%. The naturally occurring isomer is the L-form which has been found in apples and many other fruits and plants. Prepn of D- and DL-forms, and resolution of racemic mixture: McKenzie *et al.*, *J. Chem. Soc.* 123, 2875 (1923). Microbial production of L-form: Kitahara, and Abe *et al.*, U.S. pats. 2,972,566 and 3,063,910 (1961 and 1962, both to Kyowa). Configuration: J. A. Mills, W. Klyne in W. Klyne, *Progress in Stereochemistry* vol. 1 (Academic Press, New York, 1954), pp 182-183; E. L. Eliel, *Stereochemistry of Carbon Compounds* (McGraw-Hill, New York, 1962), pp 97-98; Cymerman-Craig, Roy, *Tetrahedron* 21, 1847 (1965).



DL-Form: Crystals, mp 131-132°.

D(+)-Form: Crystals, mp 101°.

L(−)-Form: *Apple acid.* Crystals from acetone or acetone + $CHCl_3$, mp 100°. Dec about 140°. $[\alpha]_D$ −2.3° (c = 8.5 in water). Freely sol in water; 1 g dissolves in 1.4 ml alcohol, 1.7 ml ether, 0.75 ml methanol, 2.3 ml propyl alcohol.

USE: *Review: Mfg. Chemist Aerosol News* 35, 56 (1964).

Mallein. Prepd as tuberculin, but using the organism *Malleomyces mallei.* Used in the mallein test, a diagnostic test for glanders in horses.

Mallow. Common or high mallow; cheeseflower. Leaves of *Malva sylvestris* L., and *M. rotundifolia* L., *Malvaceae. Habit.* Europe, Asia, naturalized in U.S. *Constit.* Pectin, tannin, coloring matter.

Malonic Acid. Propanedioic acid; methanedicarboxylic acid. $HOOCCH_2COOH$; mol wt 104.06. $C_3H_4O_4$; C 34.62%, H 3.87%, O 61.50%. Prepd from malic acid: Desmignes, *Ann.* 107, 251 (1858). Made by the interaction of monochloracetic acid and NaCN followed by hydrolysis of the resulting cyanoacetic acid: Weiner, *Org. Syn.* 18, 50 (1938). Prepn from diethyl malonate: Britton, Monroe, U.S. pat. 2,373,011 (1945 to Dow Chemical Co.); from ligneous wastes: Grangaard, U.S. pat. 2,928,868 (1960 to Kimberly-Clark Corp.); from sodium acetate: Normant, Angelo, *Bull. Soc. Chim. France* 1962, 810.

Small crystals, mp about 135° with decompn; sublimes *in vacuo.* d 1.63. One gram dissolves in 0.65 ml water, about 2 ml alcohol, 1.1 ml methanol, 3 ml propyl alcohol, 13 ml ether, 7 ml pyridine. LD_{50} i.p. in rats: 1.54 g/kg.

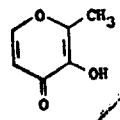
Diethyl Ester *see* Ethyl Malonate.

USE: In the manuf of barbiturates. *Human Toxicity:* Strong irritant.

Malt Extract, Powder. Contains diastase, dextrin, dextrose, protein bodies and salts from barley. *Keep dry.*

MED USE: Nutrient.

Maltol. *3-Hydroxy-2-methyl-4-pyrone;* 3-hydroxy-2-methyl-γ-pyrone; larixinic acid; Palatone; Veltol. $C_6H_6O_3$; mol wt 126.11. C 57.14%, H 4.80%, O 38.06%. Found in the bark of young larch trees (*Larix decidua* Mill.), in pine needles (*Abies alba* Mill., *Pinaceae*), in chicory, in wood tars and oils, in roasted malt. Isoln from these sources and structure: Kiliani, Bazlen, *Ber.* 27, 3115 (1894); Feuerstein, *Ber.* 34, 1804 (1901); Erdmann, Schaefer, *Ber.* 43, 2398 (1910); Reichstein, Beitter, *Ber.* 63, 824 (1930), *cf.* Peratoner, Tamburello, *Chem. Zentr.* 76, II, 680 (1905). Also obtained by alkaline hydrolysis of streptomycin salts: Schenck, Spielman, *J. Am. Chem. Soc.* 67, 2276 (1945). Synthesis from 3-hydroxy-2-(1-piperidylmethyl)-1,4-pyrone (obtained from pyromeconic acid and piperidine): Spielman, Freifelder, *ibid.* 69, 2908 (1947); Shemyakin *et al.*, *C.A.* 47, 4292a (1953). History and comparison with isomaltol: Hodge, Nelson, *Cereal Chemistry* 38, 207 (1961).



Monoclinic prisms from chloroform, orthorhombic bipyramidal crystals + monoclinic prisms from 50% alcohol, mp 161-162°. Fragant, caramel-like odor. Begins to sublime at 93°. Volatile with steam. Just acid to litmus. Absorption max 274 mμ (E_{1m} = 8400 in 0.1*N* HCl); 317 mμ (E_{1m} = 7300 in 0.1*N* NaOH). pH of 0.5% aq soln 5.3. One gram dissolves in 85 ml water. Freely sol in hot water, chloroform; sol in alcohol; sparingly sol in benzene, ether,

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and 1 ml. of hydrochloric acid, dilute to 100.0 ml. with water, and mix. Transfer 50.0 ml. of this solution into a 250-ml. Erlenmeyer flask, add 10 ml. of ammonia-ammonium chloride buffer T.S. and 12 drops of eriochrome black T.S., and titrate with 0.1 *M* disodium ethylenediaminetetraacetate until the wine-red color changes to pure blue. Each ml. of 0.1 *M* disodium ethylenediaminetetraacetate is equivalent to 12.04 mg. of $MgSO_4$.

Loss on ignition. Weigh accurately about 1 gram in a crucible, heat at 105° for 2 hours, then ignite in a muffle furnace at 450° ± 25° to constant weight.

Arsenic. A solution of 1 gram in 10 ml. of water meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. A solution of 2 grams in 25 ml. of water meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

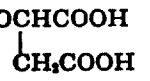
Selenium. A solution of 2 grams in 40 ml. of dilute hydrochloric acid (1 in 2) meets the requirements of the *Selenium Limit Test*, page 953.

Packaging and storage. Store in well-closed containers.

Functional use in foods. Nutrient; dietary supplement.

MALIC ACID

DL-Malic Acid; Hydroxysuccinic Acid



$C_4H_6O_5$ **Mol. wt. 134.09**

DESCRIPTION

White or nearly white, crystalline powder or granules having a strongly acid taste. One gram dissolves in 0.8 ml. of water and in 1.4 ml. of alcohol. Its solutions are optically inactive.

SPECIFICATIONS

Assay. Not less than 99.5 percent of $C_4H_6O_5$.

Melting range. Between 130° and 132°.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Fumaric acid. Not more than 0.5 percent.

Heavy metals (as Pb). Not more than 20 parts per million (0.002 percent).

Lead. Not more than 10 parts per million (0.001 percent).

Maleic acid. Not more than 0.05 percent.
Residue on ignition. Not more than 0.1 percent.
Water-insoluble matter. Not more than 0.1 percent.

TESTS

Assay. Dissolve about 2 grams, accurately weighed, in 40 ml. of recently boiled and cooled water, add phenolphthalein T.S., and titrate with 1 *N* sodium hydroxide to the first appearance of a faint pink color which persists for at least 30 seconds. Each ml. of 1 *N* sodium hydroxide is equivalent to 67.04 mg. of $C_4H_6O_5$.

Melting range. Determine as directed in the general procedure, page 931.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Fumaric and maleic acids

Buffer Solution A. Dissolve 74.5 grams of potassium chloride in 500 ml. of water in a 1000-ml. volumetric flask, add 100 ml. of hydrochloric acid, and dilute to volume with water.

Buffer Solution B. Dissolve 171.0 grams of dibasic potassium phosphate, $K_2HPO_4.3H_2O$, in 1000 ml. of water, and add monobasic potassium phosphate, KH_2PO_4, until the pH is exactly 7.0.

Maxima Suppressor. Dissolve, with the aid of a magnetic stirrer, 1 gram of gelatin in 65 ml. of hot, boiled water, and, after cooling, add 35 ml. of anhydrous ethanol as a preservative.

Standard Solution. Weigh accurately about 20 grams of the sample, 100 mg. of fumaric acid of the highest purity available, and 10 mg. of maleic acid of the highest purity available, and transfer into a 500-ml. volumetric flask. Add 300 ml. of sodium hydroxide T.S. and a few drops of phenolphthalein T.S., and then continue the neutralization with sodium hydroxide T.S. to a faint pink color that persists for at least 30 seconds. Dilute to volume with water, and mix.

Sample Solution. Transfer about 4 grams of the sample, accurately weighed, into a 100-ml. volumetric flask, and dissolve in 25 ml. of water. Add phenolphthalein T.S., and neutralize with sodium hydroxide T.S. as directed for the *Standard Solution*. Dilute to volume with water, and mix.

Procedure. Transfer 25.0-ml. portions of the *Sample Solution* into separate 100-ml. volumetric flasks. Dilute one flask (*Sample A*) to volume with *Buffer Solution A*. To the other flask (*Sample B*) add 50 ml. of *Buffer Solution B*, and dilute to volume with water.

Rinse a polarograph cell with a portion of *Sample A*, add a suitable volume of the solution to the cell, immerse it in a water bath regulated at 24.5° to 25.5°, add 2 drops of the *Maxima Suppressor*, and then de-aerate by bubbling nitrogen through the solution for at least 5 minutes. Insert the dropping mercury electrode (negative polarity) of a suitable polarograph, adjust the current sensitivity as necessary,

and record the polarogram from −0.1 to −0.8 volt at the rate of 0.2 volt per minute, using a saturated calomel electrode as the reference electrode. Transfer 25.0 ml. of the *Standard Solution* into a 100-ml. volumetric flask, and dilute to volume with *Buffer Solution A*. Obtain the polarogram of this standard (*Standard A*) in the same manner as directed for *Sample A*. In each polarogram, determine the height of the maleic acid plus fumaric acid wave occurring at the half-wave potential near −0.56 volt, recording that for the sample as i_u and that for the standard as i_s.

In the same manner, obtain polarograms from *Sample B* and *Standard B*, except record the polarogram from −1.05 to −1.7 volts at the rate of 0.1 volt per minute. In each polarogram, determine the height of the maleic acid wave occurring at the half-wave potential near −1.33 volts, recording that for the sample as i_u' and that for the standard as i_s'.

Calculation. Calculate the weight, in mg., of combined maleic acid and fumaric acid in the sample by the formula $500C \times i_u/(i_s - i_u)$, in which C is the concentration, in mg. per ml., of combined maleic acid and fumaric acid in the *Standard Solution*. Similarly, calculate the weight, in mg., of maleic acid in the sample by the formula $500C' \times i_u'/(i_s' - i_u')$, in which C' is the concentration, in mg. per ml., of maleic acid in the *Standard Solution*. Finally, calculate the weight of fumaric acid in the sample from the difference in these values.

Heavy metals. A solution of 1 gram in 25 ml. of water meets the requirements of the *Heavy metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Residue on ignition. Ignite 2 grams as directed in the general method, page 945.

Water-insoluble matter. Dissolve 25 grams in 100 ml. of water, and filter through a tared Gooch crucible. Wash the filter with hot water, dry at 100° to constant weight, cool, and weigh.

Packaging and storage. Store in well-closed containers.

Functional use in foods. Acidifier; flavoring agent.

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INVESTIGATIONS ON THE FLUOROMETRIC DETERMINATION OF MALIC AND SUCCINIC ACIDS IN APPLE TISSUE[1]

C. GUINN BARR[2]

(WITH THREE FIGURES)

Received December 3, 1947

Introduction

The role which the organic acids play in plant metabolism is not well understood. The work of PUCHER, VICKERY, and WAKEMAN (4, 5, 6, 7, 8, 9) on tobacco and other plant materials has opened the way for many investigations on total organic acids and on the various individual organic acids such as malic, oxalic, pyruvic, citric, and succinic. In our investigations on the acid content of apples the need was felt for a rapid and reliable method for the estimation of mixtures of succinic and malic acid. While the existing methods for the determination of the total organic acid are relatively simple, the determination of succinic acid as succinyl p-toluide was not entirely satisfactory because of the difficulty of the processing of a large number of samples through all the steps of the method.

The fluorometric spot test described by FEIGL (1) for the estimation of dicarboxylic acids seemed promising for development into a rapid quantitative method. In this method the acids are heated with resorcinol and concentrated sulfuric acid and upon addition of alkali the fluorescence in ultraviolet light is measured. FEIGL (1) states that "dicarboxylic acids with the carboxyl groups in the 1,2 or 1,4 positions or their derivatives, such as esters, anhydrides, or imides, form dyes of the fluorescein type on melting with resorcinol," and these give a vivid greenish-yellow fluorescence in alkaline solutions by daylight and a greenish-blue fluorescence in ultraviolet light.

Under the conditions described by Feigl, malic acid forms semi-aldehydes of malonic acid or its homologues which condense with resorcinol to umbelliferone or its homologues, and these fluoresce a brilliant blue in alkaline solution under the mercury vapor lamp. It was found, however, that this solution is not stable in an alkaline reaction and the intensity of the fluorescence fades upon standing in aqueous solution at room temperature. PRINGSHEIM (3) states that in general any photoluminescent substance has an absorption band in the spectral region immediately adjoining the short-wave limit of the luminescence band and even somewhat overlapping it. Red, therefore, is excited by orange light, yellow, by green, green, by blue, and violet by ultraviolet.

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It was found that fluorescein shows a brilliant green fluorescence in alkaline solution if activated by the spectral region between 420 and 520 millimicrons isolated by Corning filters 3389 and 5551. The fluorescence is still more intense in acid solution (pH 1.5 to 2.0), a fact used to advantage in the measurement of the fluorescein derivative of succinic acid in the presence of the umbelliferone formed by malic acid, since umbelliferone does not fluoresce in acid solution. These facts seemed to offer a basis for determining these acids quantitatively.

A Pfaltz and Bauer Fluorophotometer was used throughout this work for measuring intensity of fluorescence. In the determination of succinic acid the instrument is calibrated with a standard solution of fluorescein. Using Corning filters No. 3389 and 5551 for transmission of the exciting light and Corning filters No. 3486 and 3387 as the secondary filter combination, the instrument is adjusted so that the galvanometer reads 100 per cent. for the fluorescence of a slightly acid solution containing 50 micrograms of fluorescein per 100 ml.

The blue fluorescence of umbelliferone is apparently of about the same wave length as the blue fluorescent light emitted by quinine sulfate when illuminated by ultraviolet light and a standard quinine sulfate solution can, therefore, be used for setting the instrument for quantitative determinations of malic acid. A Corning filter No. 5970 is used as the primary filter and for the secondary filters in the pick-up unit, Corning filters No. 4308 and 3389 are used. The fluorophotometer is adjusted to read 100 per cent. fluorescence for a solution containing 300 micrograms of quinine sulfate per 100 ml.

In order to obtain a straight line relationship between the concentration and the intensity of fluorescence of a substance, it is necessary to determine the proper range of concentration to use. Since the fluorescence of a substance is not proportional over all ranges of concentration, there is an upper limit to the concentration which can be used. Higher concentrations do not yield correspondingly greater fluorescence and the fluorescence may actually become less with increasing concentration. This behavior becomes very important when testing the fluorescence of samples of unknown concentration.

Determination of succinic acid

The detection and quantitative estimation of succinic acid has been carried out as follows: a stock solution containing 1 mg. of succinic acid per ml. is used to make the standard solutions for the subsequent calibration curve. For the preliminary work, three concentrations of 5, 10, and 15 mg. succinic acid per 100 ml. should be used. Two-ml. aliquots of these solutions are transferred to 5-ml. beakers or crucibles and evaporated to dryness in an oven at a temperature between 70° and 100° C. The beakers containing the succinic acid residue are cooled to room temperature and the dry succinic acid is moistened with 0.04 ml. of pure concentrated sul-

furic acid. This is stirred with a glass rod and about 10 mg. of freshly sublimed resorcinol are added and again stirred thoroughly to insure complete mixing of the resorcinol with the succinic acid-sulfuric acid mixture. The beakers are then placed in an oven at 126°–130° C. for one hour to allow the reaction to go to completion. The effect of time on the development of the fluorescence is discussed in another part of this paper.

When the reaction is complete, the samples are removed from the oven and cooled to room temperature. About 2 ml. of distilled water are added to dissolve the products. The solutions are transferred quantitatively to 200-ml. volumetric flasks, made to the mark with distilled water and mixed. Ten-ml. aliquots of these solutions are then transferred to 100-ml. volumetric flasks, 2 to 4 drops of (1 + 1) sulfuric acid are added to bring the pH to 1.5 to 2.0, and diluted to the mark. Portions of these solutions are used for the fluorescence tests. There are several steps in the procedure which are rather critical. No difficulty should be encountered, however, if a uniform technic is established. A reagent blank must be run with each set of determinations. The 5-ml. beakers containing the samples are conveniently handled by setting them in a small tray which can be placed in the oven. Twenty-five or thirty samples can be easily processed at a time in this way.

Discussion

It was found that the fluorescence of the succinic acid derivative may vary appreciably according to the order and manner of adding, and amount of reagents used. For example, it is essential that the dry succinic acid sample be moistened with 0.04 ml. of pure concentrated sulfuric acid before the resorcinol is added and the intensity of the fluorescence is decreased by the use of greater amounts of sulfuric acid. A quantity of resorcinol far in excess of the theoretical amount is required to insure complete reaction of all of the succinic acid present. The resorcinol should be freshly sublimed and powdered in an agate mortar and preliminary tests made to determine the requirements under the existing conditions. A small glass spatula or spoon can be made for dispensing the resorcinol. If a large excess of resorcinol is used, the reaction products may have a dark red color after treatment at 130° C., but the sensitivity of the tests is not altered. Neither is the sensitivity affected by the presence of malic acid in concentrations up to 15 micrograms per 100 ml. When a sample containing malic acid instead of distilled water is used as a reagent blank, a slightly higher fluorescence may be obtained for the blank reading but this is taken into account in calculating the net readings.

The succinic acid derivative is reasonably stable for several hours in the final dilute acidified solution. The intensity of the fluorescence will fade appreciably, however, if the samples are allowed to stand more than 48 hours. Fading has been observed also when the samples were allowed to remain longer than two hours in the 200-ml. volumetric flasks before aliquots were taken for fluorescence readings.

TABLE I

THE EFFECT OF VARYING QUANTITIES OF SULFURIC ACID UPON INTENSITY OF FLUORESCENCE

ML. OF SULFURIC ACID USED	INTENSITY OF FLUORESCENCE*	
	SUCCINIC ACID	MALIC ACID
0.02	89.0	100.0
	92.0	97.0
0.04	93.0	99.0
	96.0	98.0
0.06	96.0	100.0
	85.0	98.0
0.08	68.0	13.0
	78.0	26.0

* The values given are per cent. of light emitted as determined by use of the Fluorophotometer.

Crystallization of the resorcinol-sulfuric acid-organic acid mixture occurred before the samples were placed in the oven at 130° C. when more than 0.04 ml. of sulfuric acid was used with the quantity of resorcinol employed. In all cases the samples which did not crystallize fluoresced with greater intensity than those which crystallized.

Data are presented in table II which are typical of the results obtained repeatedly in testing the reliability of the succinic acid measurements in pure solution. These data are the results of six different sets of determinations with from two to five replications per set. Replications are individual samples carried through all the steps in the procedure and represent 2-ml. aliquots taken from standard solutions for evaporation.

Determination of malic acid

The procedure for the determination of malic acid is essentially the same as that used for succinic acid up to the step where the fluorescence is to be measured. Two-ml. aliquots of solutions containing 5, 10, and 15 mg. of malic acid per 100 ml. are evaporated to dryness and treated with

TABLE II

FLUORESCENCE INTENSITY OF THE FLUORESCEIN DERIVATIVE OF SUCCINIC ACID CONCENTRATION OF SUCCINIC ACID IN MICROGRAMS/100 ML.

5	10	15	5	10	15
29.5	61.5	87.5	31.0	62.0	84.0
32.0	62.0	86.0	32.0	62.0	87.0
33.0	56.0	86.0	25.5	58.5	85.5
33.0	54.0	84.0	28.5	53.5	84.5
32.0	56.0	87.0	29.5	60.5	85.5
33.0	56.0	87.0	33.0	63.0	91.0
31.5	56.5	83.5	33.0	63.0	92.0
29.5	57.5	80.5	31.0	58.0	85.0
27.5	62.5	84.5			
32.0	59.0	88.0	Av. 30.91	Av. 58.9	Av. 86.02

concentrated sulfuric acid and resorcinol, heated at 130° C. for 1 hour, transferred to 200-ml. volumetric flasks and made to the mark.

In the determination of the fluorescence of the malic acid derivative, 10-ml. aliquots of the solutions from the 200-ml. volumetric flasks are transferred to 100-ml. volumetric flasks and 1 ml. of 0.1 N NaOH are added before diluting to the mark. This brings the pH of the solution to about 10.5 at which maximum fluorescence is obtained. The fluorescence of these solutions must be measured without delay to avoid fading since the umbelliferone derivative is not stable at room temperature for more than an hour. Fluorescence intensities of the derivatives of succinic and malic acid in various concentrations are given in table III and the data are shown graphically in figure 1.

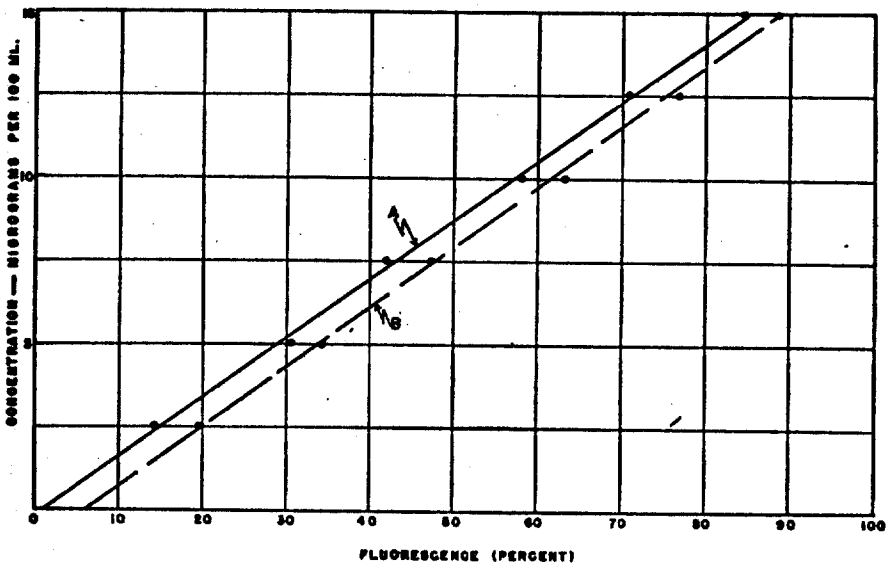

FIG. 1. The relation of succinic and malic acid concentration to the fluorescence of their resorcinol derivatives. (A) Succinic acid. (B) Malic acid.

DISCUSSION

At the outset it was found that alkaline solutions of the malic acid derivative appeared to be highly unstable. Fluorescence values were not uniform for replicate samples and the intensity decreased rapidly upon standing. If the samples were allowed to remain in a strongly alkaline reaction for 24 hours, the fluorescence dropped to about one-third of the original value and after two or three days the fluorescence was almost completely lost. The fluorescence could be restored and sometimes increased, however, by adding sulfuric acid to bring the pH to about 2 and again shifting the reaction back to pH 10 to 11 by the addition of sodium hydroxide solution. Further study showed that the rate of fading was somewhat dependent upon the concentration of the sodium hydroxide solution used. The addition of strong alkali (2 drops of 20 per cent. NaOH)

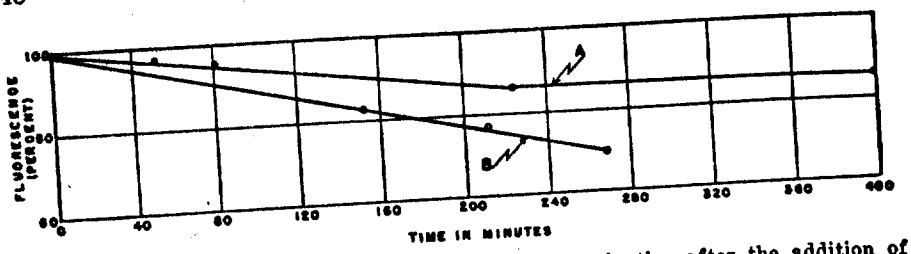

FIG. 2. Fading in fluorescence of the malic acid derivative after the addition of N alkali. (A) 1 ml. of 0.1 N NaOH. (B) 0.04 ml. of 20% NaOH.

caused a very rapid decay of fluorescence (a loss of about 30% in intensity after 4.5 hours). One ml. of 0.1 N sodium hydroxide added to the aliquot before making to volume, however, resulted in a loss of about 15 per cent. after 6.5 hours (Fig. 2). Fluorescence measurements can be made, therefore, on dilute solutions of the malic acid derivative in weakly basic reaction with minimum chance for error if the readings are made within a few minutes after the addition of the alkali.

Acid solutions of the malic acid derivative are stable indefinitely and it may be convenient, therefore, to pipette the appropriate aliquots into volumetric flasks, make to volume and mix, and add 2 to 4 drops of 0.1 N sodium hydroxide dropwise to a portion of the sample in the fluorometer cell just before making the reading.

Fluorescence of the succinic and malic acid derivatives as affected by time of heating

FEIGL (1) states that positive results for dicarboxylic acids may be obtained in the spot test technic by conversion into dyes of the fluorescein type upon heating the resorcinol melt at 130° C. for five minutes. It was found that the heating time of five minutes used in Feigl's spot test led to erratic results indicating an incomplete conversion in dyes of the fluorescein type. We determined that the maximum fluorescence is obtained after

TABLE III

THE EFFECT OF CONCENTRATION ON INTENSITY OF FLUORESCENCE

CONCENTRATION IN MICROGRAMS/100 ML.	INTENSITY OF FLUORESCENCE OF CONDENSATION PRODUCT	
	SUCCINIC ACID*	MALIC ACID†
2.5	14.0	19.3
5.0	30.7	34.3
7.5	41.9	47.5
10.0	58.5	63.3
12.5	71.0	76.8
15.0	84.9	89.0

* Fluorescein derivative of succinic acid, pH 1.5. Wave length of activating light between 420–520 millimicrons.

† Umbelliferone derivative of malic acid, pH 10.5. Wave length of activating light between 320–420 millimicrons.

TABLE IV

INTENSITY OF FLUORESCENCE AS AFFECTED BY TIME OF HEATING

MINUTES HEATED	INTENSITY OF FLUORESCENCE	
	SUCCINIC ACID	MALIC ACID
10 min.	60.5*	12.0†
20 min.	64.5	23.5
30 min.	85.0	24.0
45 min.	86.5	47.4
60 min.	98.5	79.0
75 min.	90.5	76.7
90 min.	92.0	77.5
105 min.	95.0	78.7
120 min.	92.0	79.3
		83.5
		92.0
		93.0
		97.0
		97.0
		93.5
		86.5
		87.5
		89.5

* Heated with 0.04 ml. of sulfuric acid. † Heated with 0.06 ml. of sulfuric acid.

heating the mixture for one hour at 130° C. The results of these experiments are assembled in table IV.

Detection of succinic and malic acids in pure and in mixed solutions

Using solutions of known concentration, a calibration curve, for use in the estimation of unknown amounts of succinic and malic acid, should be constructed with each set of determinations. In quantitative fluorometric analysis, the accuracy of measurement decreases as the values approach either extreme of the galvanometer scale. Discrepancies, therefore, are to be expected in detecting organic acids in concentrations less than 5 micro-

TABLE V

DETERMINATION OF SUCCINIC ACID IN MIXED SOLUTIONS OF MALIC AND SUCCINIC ACIDS

AMT. OF ORGANIC ACID PRESENT (MICROGRAMS/100 ML.)		NET FLUORESCENCE	MICROGRAMS OF SUCCINIC ACID DETERMINED	VARIATION FROM THEORETICAL (MICROGRAMS)
SUCCINIC	MALIC			
0.0	5.0	0.0	0.0	———
1.0	5.0	6.1	1.0	0.0
2.0	5.0	10.0	1.7	−0.3
4.0	5.0	21.0	3.6	−0.4
5.0	5.0	24.5	4.2	−0.8
0.0	10.0	0.0	0.0	———
1.0	10.0	5.0	0.8	−0.2
2.0	10.0	9.0	1.5	−0.5
4.0	10.0	24.0	4.1	+0.1
5.0	10.0	31.0	5.3	+0.3
10.0	10.0	55.0	9.4	−0.6
0.0	15.0	0.0	0.0	———
1.0	15.0	8.3	1.4	+0.4
2.0	15.0	14.0	2.4	+0.4
4.0	15.0	25.0	4.2	+0.2
5.0	15.0	31.0	5.3	+0.3
10.0	15.0	59.0	10.0	0.0
15.0	15.0	87.0	15.0	0.0

grams per 100 ml. when the instrument is calibrated for detection of quantities ranging between 5 and 15 micrograms per 100 ml.

Various amounts of succinic acid were added to three different concentrations of malic acid. Results of the succinic acid determinations are shown in table V. Estimations of succinic acid within one microgram per 100 ml. were made and apparently the presence of malic acid in the proportions used had no important effect on the accuracy of the determinations.

Malic acid in pure solution can be determined with reasonable accuracy. The presence of succinic acid and other organic acids that yield substances which show a bluish-green fluorescence under ultraviolet light after treatment with resorcinol and sulfuric acid increase slightly the apparent malic acid values. Some of this fluorescence is screened out, however, by the secondary filters. The fluorescence of the succinic acid derivative, furthermore, is quenched materially in the presence of comparable concentrations of umbelliferone. In this connection it should be pointed out also that the fluorescence of the fluorescein, produced by small amounts of succinic acid which may be present, is not effectively excited by the relatively low light requirements of the activating beam used for malic acid determinations (table VI).

TABLE VI

THE EFFECT OF VARYING QUANTITIES OF SUCCINIC ACID UPON THE INTENSITY OF FLUORESCENCE OF MALIC ACID DERIVATIVES

AMT. OF ACID PRESENT (MICROGRAMS/100 ML.)		INTENSITY OF FLUORESCENCE FOR MALIC ACID (NET)
MALIC	SUCCINIC	
5	0	35.5
5	1	32.0
5	2	34.5
5	4	30.0
5	5	34.0
5	10	35.0
5	15	31.0
10	0	62.5
10	1	65.0
10	2	61.5
10	4	65.0
10	5	64.5
10	10	61.0
10	15	64.0
15	0	88.5
15	1	88.0
15	2	89.0
15	4	90.0
15	5	89.0
15	10	88.0
15	15	91.0

Results of studies on the recovery of malic acid in the presence of varying amounts of succinic acid are presented in table VII.

TABLE VII

DETERMINATION OF MALIC ACID IN MIXED SOLUTIONS OF SUCCINIC AND MALIC ACIDS

AMT. OF ORGANIC ACID PRESENT (MICROGRAMS/100 ML.)		NET FLUORESCENCE	MICROGRAMS OF MALIC ACID DETERMINED	VARIATION FROM THEORETICAL (MICROGRAMS)
MALIC	SUCCINIC			
5.0	0.0	0.0		
5.0	2.0	16.4	2.6	+0.6
5.0	4.0	24.4	3.9	−0.1
5.0	5.0	30.9	4.9	−0.1
10.0	0.0	0.0		
10.0	2.0	18.0	2.8	+0.8
10.0	4.0	23.0	3.7	−0.3
10.0	5.0	27.6	4.4	−0.6
10.0	10.0	59.0	9.4	−0.6
15.0	0.0	0.0		
15.0	2.0	15.0	2.4	+0.4
15.0	4.0	25.0	4.0	0.0
15.0	5.0	31.5	5.0	0.0
15.0	10.0	65.3	10.4	+0.4
15.0	15.0	88.0	14.1	−0.9

Detection of succinic and malic acids in plant extracts

A method for determining succinic and malic acids in pure solutions and in solutions of mixed pure organic acids to be of value must be suitable for the analysis of extracts of biological tissue. The organic acid fraction was extracted from apple tissue by a method essentially as described by ISAACS and BROYER (2). Aliquots of 2, 5, and 10 ml. of uniform samples of the extract were diluted to 100 ml. and designated as samples A, B, and C. Determinations of malic acid were made by the fluorometric method described above and compared with the results obtained by the oxidation method of Pucher and Vickery. The results are shown in table VIII.

Results of the fluorometric method of analysis for the determination of succinic and malic acid in apple tissue extracts containing known additional amounts of these acids are presented in table IX. Our studies

TABLE VIII

ESTIMATION OF MALIC ACID IN APPLE TISSUE EXTRACT

SAMPLE	MALIC ACID CONTENT IN MG./ML. OF ORGANIC ACID EXTRACT*	
	FLUOROMETRIC METHOD	OXIDATION METHOD
A	1.80	1.90
B	2.16	2.00
C	2.56	2.45

* Each ml. organic acid extract represents 2.00 gm. fresh apple tissue.

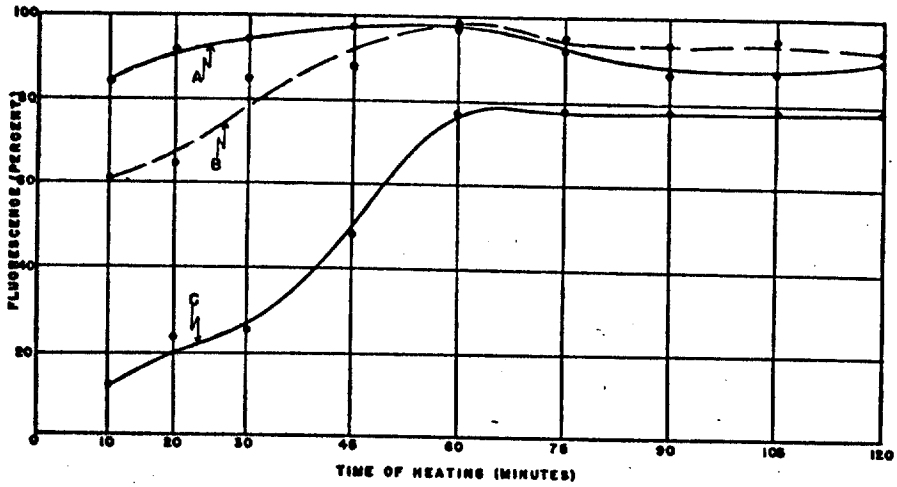

FIG. 3. Intensity of fluorescence as affected by time of heating. (A) Malic acid—using 0.04 ml. of sulfuric acid in the reaction. (B) Succinic acid—using 0.04 ml. of sulfuric acid in the reaction. (C) Succinic acid—using 0.06 ml. of sulfuric acid in the reaction.

showed the estimations for malic acid can be made with about the same degree of accuracy as those for succinic acid. Most accurate results are obtained when the fluorometric readings are between 30 and 80 on the scale of the galvanometer.

Determinations were made by the fluorometric method of succinic and malic acid in tobacco and corn leaf-tissue. The succinic acid content of

TABLE IX

DETERMINATION OF SUCCINIC AND MALIC ACIDS IN EXTRACTS OF APPLE TISSUE

NET FLUORESCENCE	MICROGRAMS/100 ML. PRESENT	ADDED	TOTAL PRESENT	DETERMINED	VARIATION FROM THEORETICAL (MICROGRAMS)
	SUCCINIC ACID				
7.0	1.0	0.0	1.0	1.2	+0.2
17.0	1.0	2.0	3.0	2.9	−0.1
30.0	1.0	4.0	5.0	5.1	0.0
34.5	1.0	5.0	6.0	5.8	−0.2
60.3	1.0	10.0	11.0	10.2	−0.8
88.0	1.0	15.0	16.0	15.0	−1.0
	MALIC ACID MICROGRAMS /100 ML.				
35.8	5.0	0.0	5.0	5.4	+0.4
43.5	5.0	2.0	7.0	6.9	−0.1
47.0	5.0	2.5	7.5	7.5	0.0
61.5	5.0	5.0	10.0	9.8	−0.2
74.0	5.0	7.5	12.5	12.1	−0.4

tobacco leaves was 0.10 per cent. and that of corn 0.05 per cent. (fresh weight basis); whereas, the acid content estimated as malic was 1.02 and 0.15 per cent. (fresh weight basis) for tobacco and corn leaves, respectively.

The malic acid content of plant tissue varies materially with the species and with the part of the plant studied. Leaf tissue, in general, is relatively low in malic acid content, while fruits, especially apples, usually contain appreciable quantities. Furthermore, succinic acid normally constitutes only a small percentage of the total organic acid content and the presence of naturally occurring succinic acid will ordinarily have practically no effect on the malic acid determinations. The malic acid derivative shows virtually no fluorescence in acid reaction when measurements are made for succinic acid and relatively high concentrations of malic acid may be present without interference.

The presence of citric acid and other substances which yield compounds of the umbelliferone type when treated with resorcinol and sulfuric acid is troublesome in evaluating the malic acid content. Preliminary investigations have shown a direct relationship between fluorescence and concentrations of citric acid in terms of the resorcinol derivative. The fluorometric method is suitable, however, for malic acid when only malic, or malic and succinic are present. In the presence of citric acid the total amount of both malic and citric may be determined and the malic acid content calculated by difference after determining the percentage of citric acid by some other means. The author has found the fluorometric method as described herein to be reliable for the determination of succinic acid. Although the fluorometric procedure was developed primarily for the determination of succinic and malic acid in apple tissue, results obtained in this investigation indicate that the method should be adaptable to other tissues.

Summary

Succinic acid and malic acid when heated with resorcinol and sulfuric acid yield compounds which show intense fluorescence under specific conditions. Fluorometric separation of the succinic- and malic-acid derivatives has been accomplished by controlling the exciting light and the reaction of the test solution. Dilute solutions of the succinic acid derivatives show an intense green fluorescence in acid reaction when illuminated by light of wave length 420 to 520 millimicrons. The malic acid derivative shows virtually no fluorescence under these conditions but fluoresces a brilliant blue in basic solution under ultraviolet light of 320 to 420 millimicrons in length.

A photometric method based on these properties has been developed for the quantitative determination of these acids in mixed solutions, and in certain plant tissues.

The author expresses his thanks to Dr. Erwin J. Benne of the Agricultural Chemistry Department for many helpful suggestions in the prepara-

tion of the manuscript. Kind thanks are also due Miss Selma Bandemer for her counsel during the early stages of this investigation.

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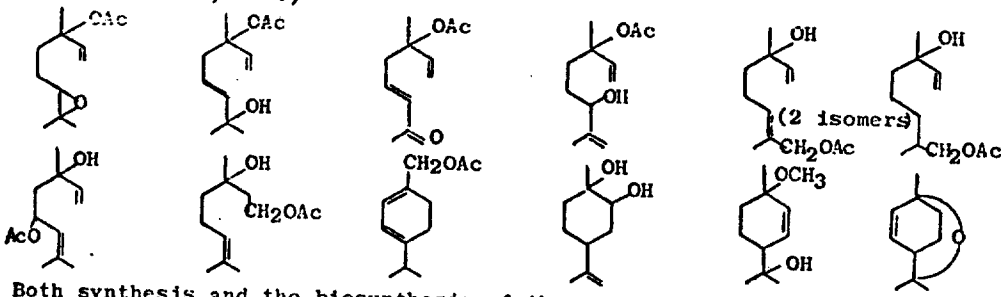
37. OCCURRENCE OF BIFUNCTIONAL MONO-TERPENIC COMPOUNDS IN BERGAMOT OIL. Braja D. Mookherjee, International Flavors & Fragrances (IFF-R&D), 1515 Highway 36, Union Beach, N. J. 07735.

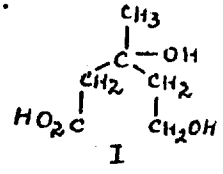
Bergamot oil was subjected to molecular distillation to obtain volatile and non-volatile materials. The volatile materials were fractionated into individual components by the combination of both column and gas-liquid chromatography. The pure components thus isolated were analyzed by IR, NMR and MS. By this technique a host of new bifunctional mono-terpenic compounds were identified, viz.,



Both synthesis and the biosynthesis of these compounds will be discussed.

38. ROLE OF GLYCINE IN TERPENE BIOSYNTHESIS. Ajay K. Bose and K.S. Khanchandani, Department of Chemistry and Chemical Engineering, Stevens Institute of Technology, Hoboken, N.J. 07030.

Mevalonic acid (I) is generally considered to be a primary precursor of terpenes. However, very often the poor incorporation of I in some terpenes has been ascribed to difficulties of transport across cellular membranes. Since amino acids are known to be easily transported through cell barriers, we have examined the possible role of some amino acids in the biosynthesis of the sester-terpene ophiobolin B (II). The fungus cochliobolus miyabeanus was grown in a chemically defined medium containing ^{14}C and ^{13}C labeled compounds. The labeled ophiobolin B (L. Canonica et al, Tetrahedron Letters, 275 (1968)) obtained this way was examined by mass spectral and other methods. We have found that glycine is incorporated into II more efficiently than mevalonic acid and that the incorporation of the methylene carbon of glycine is about 30 times higher than that of the carboxy carbon. The implications of these and other finding will be discussed.



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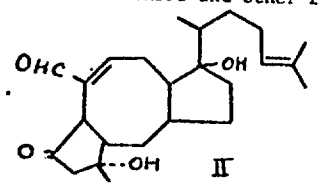

II

TUESDAY AFTERNOON - SECTION C - SYMPOSIUM ON PROTEIN FROM PETROLEUM. THE COMPETITION - Joint with Division of Petroleum Chemistry & Microbial Chemistry and Technology - Abstracts in Section PETR

WEDNESDAY MORNING - SECTION A - GENERAL - K. Morgareidge, Presiding

39. THE DETERMINATION OF ORGANIC ACIDS IN PLANT AND FOOD PRODUCTS. W. R. Harvey, R. W. Hale, and R. M. Ikeda. Philip Morris, Inc., Research Center, P.O. Box 3D, Richmond, Virginia 23206.

A simple method for the determination of malic, citric and oxalic acids in tobacco leaf, fruits, and vegetables is presented. A dry sample is simultaneously extracted and esterified with 10% sulfuric acid in absolute methanol. The methyl esters formed from this reaction are extracted into chloroform and determined gas chromatographically. The procedure is applicable for a wide variety of samples. Good precision and recoveries are obtained. Other organic acids could be determined via this procedure.

CHROM. 5253

A mixed silica gel–cellulose support for thin-layer chromatography of non-volatile organic acids

Thin-layer chromatography has been used in this laboratory in a study of the non-volatile organic acids in guava[1]. Difficulties were encountered in obtaining adequate resolution of the acids on cellulose and on silica gel supports. The effectiveness of mixtures of the two materials for TLC supports was investigated and is the subject of this note. Silica gel–cellulose supports have been used with varying degrees of success for the separation of amino acids[2,3]. We found that a silica gel–cellulose mixture eliminated the streaking found on cellulose and showed better resolution of acid spots than did silica gel alone.

Materials and methods

Mixtures of MN Silica Gel G and Cellulose MN 300 (Macherey, Nagel & Co.)* were spread on glass plates 20 × 20 cm with a Desaga spreader. Silica gel was mixed at 20, 30, 40, 50, 60, and 70% levels with cellulose. Total volume of water added to 30 g of the mixture was 6 parts of water to each part of cellulose (w/w) plus 2 parts of water to each part of silica gel (w/w). The silica gel–cellulose mixtures were blended for 1 min in a Waring blendor, spread at a thickness of 300 μ. Coated plates were left to dry and set overnight at ambient temperature and activated at 110° for 1 h. Plates were cooled and stored in a desiccator until used.

Three solvent systems were used. Solvent (I) anhydrous diethyl ether–88% formic acid–water (20:5:3); (II) *n*-butanol–88% formic acid–water (4:1:5); (III) benzyl alcohol–*tert.*-butanol–isopropanol–88% formic acid–water (24:8:8:1:8).

Mixtures of known organic acid solutions (50 mg/ml in 95% ethanol) were applied in 1-μl quantities to the plates under a stream of warm air. The plates were allowed to cool to ambient temperature and then were developed to a height of 10 to 12 cm. After development, the plates were dried overnight at ambient temperature. Bromphenol Blue reagent (0.04% in 95% ethanol and 0.05% sodium acetate) was used to detect the acids.

Results and discussion

The use of silica gel–cellulose mixtures corrected major problems which arose from use of either support individually. Mixtures with increasing percentages of silica gel progressively eliminated the streaking found with use of cellulose. In addition, supports of cellulose mixed with silica gel permitted resolution of spots previously showing poor resolution[3] with silica gel as the sole ingredient. Plates with lower percentages of silica gel proved easier to handle and spot, the support layer being less brittle and more adhesive than higher percentage silica gel supports.

In order to determine the most satisfactory percentage of silica gel a mixture was sought which gave the best resolution, least streaking and easiest plate handling.

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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The 60% silica gel–40% cellulose mixture proved to be the most satisfactory support. Plates required no special handling, and no difficulties were encountered during application of samples. Spots were distinct and compact with no streaking. In addition, the incorporation of 60% silica gel with cellulose gave the best separation in terms of giving the maximum differences in R_F values between malic and citric and between ascorbic and tartaric acids in comparison to those from 20, 40, 50, and 70% levels of silica gel with cellulose.

TABLE I

R_F VALUES (× 100) OF ORGANIC ACIDS ON 60% SILICA GEL IN CELLULOSE DEVELOPED WITH THREE SOLVENT SYSTEMS

Acid	*Solvents*		
	I	*II*	*III*
Fumaric	97	95	92
Adipic	97	92	91
Glutaric	94	90	94
Succinic	88	88	87
Lactic	84	77	78
Oxalic	82	69	70
Malic	59	61	64
Citric	51	57	55
Pyroglutamic	61	52	72
Ascorbic	31	45	58
Tartaric	36	36	46
Quinic	20	26	37
Galacturonic	07	09	18

Several non-volatile organic acids that are commonly found in plant material were spotted on thin-layer plates of 60% silica gel–40% cellulose and developed in three different systems (Table I). The elution order was the same under the three different solvent systems with the exception of glutaric and pyroglutamic acids.

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Collaborative Study of a Method for Determination of *l*-Malic Acid in Grapes and Grape Products

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The polarimetric method for the determination of *l*-malic acid in grape juice and grape products has been further modified by increasing the amount of potassium acetate used, adjusting the alcohol wash concentration, and utilizing a more efficient filtration for the removal of interfering tartrate salts. Collaborators reported acceptable results and no difficulties were reported. It is recommended that the method as modified be adopted as official first action for the determination of *l*-malic acid in grape juice and grape products.

A collaborative study was recently reported of a polarimetric method for the determination of *l*-malic acid in fruits and fruit products (1). Satisfactory results were obtained for all fruits examined except grape juice and grape preserves. The method used was a modification of techniques previously developed by Hartmann (2) and changes published by Vandercook (3). As a result of this study, the method was adopted as official first action (4) for the determination of *l*-malic acid in fruits and fruit products with the cautionary note "(Not applicable to grapes and grape products.)".

Further study indicated that modification of the method by increasing the amount of potassium acetate used to precipitate tartrates would give satisfactory results, and the modified method was subjected to collaborative study on grape juice and grape preserve samples. Good results were reported (5) by all collaborators on grape juice; however, two collaborators reported low results on grape preserves.

Modifications subsequently found necessary to obtain satisfactory results in the analysis of grapes and grape products include: further increasing the amount of potassium acetate used to insure complete removal of the interfering tartrate ion, allowing the mixture to stand overnight to permit tartrate crystal growth and facilitate filtration, and adjusting the alcohol content to 85% for all washings of the lead salts.

METHOD

Preparation of Sample

(a) *Grape juice.*—Weigh 125 g into 500 ml vol. flask, add 1.0 ml satd KOAc soln and 200 ml absolute alcohol, and mix. Dil. to vol. with absolute alcohol, mix, and let stand overnight. Filter thru Whatman No. 40 paper, or equiv.

(b) *Grape preserves and other high-sugar content grape products.*—Comminute and weigh 125 g into 500 ml beaker. Add 1.0 ml satd KOAc soln, 50 ml H_2O, and 200 ml absolute alcohol. Mix and quant. transfer to 500 ml vol. flask with absolute alcohol. Dil. to vol. with absolute alcohol, mix, and let stand overnight. Filter thru Whatman No. 40 paper, or equiv.

(c) *Grapes.*—Comminute and weigh 125 g into 500 ml beaker. Proceed as in (a) except omit addn of 50 ml H_2O.

Determination

Transfer 200 ml aliquot filtrate to 8 oz wide-mouth bottle, 5 × 2¼″ od, contg magnetic stirring bar. Potentiometrically titr. 10 ml remaining filtrate to pH 8.4 with 0.1*N* NaOH. Calc. ml NaOH necessary to neutze 200 ml aliquot. Add to bottle 0.6 ml satd $Pb(OAc)_2$ soln for each ml 1*N* NaOH calcd to neutze 200 ml aliquot. Stir 10 min on magnetic stirrer and centrif. 6 min at 1500 rpm. Test supernatant for complete pptn with few drops satd $Pb(OAc)_2$ soln. Decant and wash ppt by stirring 5 min with 200 ml 85% alcohol. Centrf. 5 min, decant, and repeat washing step once. Add 25 ml H_2O to ppt and mix well to slurry. Proceed as in "Changes in Methods," *This Journal* 51, 465–466 (1968), *Determination*, starting with "Use pH meter and adjust pH to 1.5 with H_2SO_4 (1+9)."

Results and Discussion

Table 1 gives the results of the analyses of six samples of grape juice, jelly, and preserves by seven collaborators and shows the averages and standard deviations. All results were satisfactory and the collaborators reported no difficulties with the method. Values obtained by an eighth collaborator were considered invalid because the procedure was not followed.

Table 2 gives recovery data for the paired sam-

Table 1. Collaborative results for *l*-malic acid content of various grape products

	Per Cent *l*-Malic Acid, Sample No.:[a]					
Coll.	1	2	3	4	5	6
1	0.34	0.42	0.13	0.22	0.18	0.26
2	0.31	0.40	0.11	0.21	0.17	0.25
3	0.32	0.41	0.11	0.20	0.17	0.25
4	0.33	0.42	0.10	0.21	0.15	0.23
5	0.31	0.42	0.11	0.20	0.17	0.25
6	0.33	0.43	0.11	0.20	0.16	0.23
7	0.30	0.40	0.10	0.20	0.16	0.25
Av.	0.32	0.41	0.11	0.21	0.17	0.25
Std dev.	0.01	0.01	0.01	0.01	0.01	0.01

[a] 1, three parts Concord grape juice plus one part water; 2, undiluted Concord grape juice; 3, grape jelly; 4, grape jelly plus 0.1% *l*-malic acid; 5, grape preserves; 6, grape preserves plus 0.08% *l*-malic acid.

ples in Table 1 in which the second sample of each pair contained an added known amount of *l*-malic acid. Recoveries ranged from 95 to 100% for grape jelly and 96 to 104% for grape preserves.

Recommendations

It is recommended that this method be adopted as official first action for the determination of *l*-malic acid in grapes and grape products and that the cautionary note (4, 5) be removed from the method.

Table 2. Per cent recoveries for paired samples: second sample of each pair contains added known amount of *l*-malic acid

	Per Cent Recovery, Collaborator No.:						
Sample	1	2	3	4	5	6	7
4	97	100	96	96	95	95	100
6	100	100	100	100	96	104	100

Acknowledgments

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The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee D, and were adopted by the Association; their reports will appear in *This Journal* 53, March 1970.

This report of the Associate Referee, E. Fernandez-Flores, was presented at the 83rd Annual Meeting of the Association of Official Analytical Chemists, Oct. 13–16, 1969, at Washington, D.C.

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MANOMETRIC DETERMINATION OF l(—) MALIC ACID IN GRAPE MUSTS AND WINES[1]

GEORGE F. KOLAR[2]

INTRODUCTION

A convenient analytical method for the determination of malic acid has long been sought in winemaking research. The commonly used technique of permanganate oxidation (7) is lengthy and tedious and, unless great care in manipulation and standardization of experimental conditions is taken, the estimations of malate are inconsistent.

The quantitative conversion of l(—) malate into lactate and carbon dioxide by an inducible enzyme from washed resting cells of *Lactobacillus arabinosus* (Strain 17 - 5) was first described by Korkes and Ochoa in 1948 (3), and the enzyme system responsible studied by Ochoa and others (6, 1, 2). The decarboxylation of l(—) malic acid was then investigated by Nossal who pointed out its analytical potential (4, 5).

In the overall reacation

$$HOOC—CH_2—CHOH—COOH \longrightarrow CH_3—CHOH—COOH + CO_2$$

the volume of carbon dioxide evolved is a direct measure of l(—) malate present in the sample. However, the possibility of an additional evolution of carbon dioxide from sugar and other grape constituents in musts and the inhibition of the enzyme system by ethanol in wine, made it necessary to investigate the reaction further before the method could be applied to these products.

The following paper describes this work and gives details of a practical antlytical method for the determination of l(—) malic acid in grape musts and wines.

MATERIALS AND METHODS

Culturing Procedure: A subculture of *Lactobacillus arabinosus*, obtained from the Department of Biochemistry, University of Adelaide, was maintained on a liquid medium containing 2 per cent glucose, 2 per cent dl malic acid, 1 per cent yeast extract (Difco), 1 per cent extract or nutrient broth (Difco), 1 per cent sodium acetate trihydrate, 0.1 per cent dipotassium hydrogen orthophosphate; 5 mls of Salts "B" Mixture of Wright and Skeggs were added per liter of medium (9). This mixture contained 40 mg $MgSO_4.7\ H_2O$, 2 mg NaCl, 2 mg $MnSO_4.4H_2O$ and 2 mg $FeSO_4.7\ H_2O$ in 1 ml. The pH of the medium was adjusted by 5.5 by addition of sodium hydroxide, and the complete preparation sterilized by heating in a steamer for 20 minutes on 2 consecutive days. To keep the organism in vigorous growth, particularly before starting analyses, it was subcultural in Kimble test tubes of the above medium every second day and the culture kept at 30°C in an incubator. A stock culture of *Lactobacillus arabinosus* can be grown on slopes or stabs of nutrient agar (Difco) and kept in a refrigerator for several months without appreciable loss of viability. A freeze-dried culture was found to be viable after more than one year's storage at room temperature. Bulk cultures of the organism were grown for 24 hours at 30°C on the above described medium in 2 liter conical flasks. They were inocculated with 1-2 per cent of vigorously growing subculture, approximately 4 tubes per liter of medium. The cells (about 3 g wet weight from 1 liter of medium) were harvested by centrifuging, the culture medium drawn off by means of a pump, and the cells washed twice with 50 ml of distilled water. The harvested cells were suspended in 30 ml 1 N KCl to which 0.45

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g $MnCl_2$ and 0.30 g KH_2PO_4 had been added. To prevent deterioration and growth of contaminating microörganisms, the cell suspension, acetate buffer and the reference solution of malic acid was stored in a refrigerator at all times when not in use. This volume of cell suspension was sufficient for about 150 determinations.

Analytical Technique: Malic acid was determined in a standard type Warburg apparatus fitted with 15 ml flasks and calibrated to a reference point of 10 cm on the manometers to extend the range of the instrument. The gas phase was air and the temperature of the bath 25°C.

Reagents: 1) A 3 M acetate buffer of pH 5 prepared by dissolving 27.2 g sodium acetate trihydrate and 6 g glacial acetic acid and making up the volume to 100 ml with distilled water. 2) 0.2 per cent solution of l(—) malic acid. For accurate work the purity of malic acid was checked by paper chromatography and the concentration by titration against standard alkali. 3) A suspension of *Lactobacillus arabinosus* cells prepared as already described.

Procedure: The working procedure involved pipetting 0.1 ml of acetate buffer into the main compartment and side arm of each Warburg flask, and 0.5 ml of sample into the main compartment. This was followed by 2 ml of distilled water which was run out of a pipette down the wall of the flask to wash off any adhering substrate. The control was charged with 0.5 ml of 0.2 per cent l(—) malic acid solution and the side arm of each flask was carefully filled with 0.2 ml of *Lactobacillus arabinosus* cell suspension. Following temperature equilibration the cell suspension was mixed with the substrate to start the reaction.

Manometric readings were taken after 30 minutes as the evolution of carbon dioxide was usually complete by this time. However, to ensure complete decarboxylation the reaction was allowed to continue and the manometers were read again after 5 and 10 minutes. The amount of malate in the sample was calculated from the recorded readings, corrected for thermobar change, and the appropriate flask constant. Johnson's correction factor for the retention of carbon dioxide in buffers was applied in computing the flask constants (8), and the calculations were based on the simple relationship that 1 μmole of l(—) malic acid liberates 22.25 μl carbon dioxide (1 mg l(—) malic acid liberates 166 μl carbon dioxide). Under the experimental conditions the determination of malate was always within 3 per cent of the theoretical value and usually higher.

RESULTS AND DISCUSSION

Specificity of the method: Nossal (4) showed that the decarboxylation reaction of l(—) malic acid by resting cells of *Lactobacillus arabinosus* was accelerated by a combination of glucose, manganous chloride and potassium dihydrogen phosphate, the optimum concentrations being 10^{-4} — 10^{-1} M, 10^{-2} and 10^{-1} M, respectively. High concentration of fermentable sugars in musts, however, raised the possibility of additional carbon dioxide evolution caused by fermentation concomitant with malate decarboxylation. Addition of glucose to the sample did not cause an increase of carbon dioxide evolution and the experimental results are listed in table 1.

Further studies by Nossal (5) revealed that the only substrates undergoing de-

TABLE 1

The Determination of l(—) Malic Acid in a Standard Aqueous Solution Containing 1 mg l(—) Malic Acid in the Presence of d + Glucose at 25° C

Glucose added (mg)	Malic acid determined (mg)
None	0.95
....	0.94
0.5	0.97
0.5	0.97
1.0	0.96
1.0	0.95
25.0	0.94
25.0	0.92
50.0	0.92
50.0	0.95
100.0	0.96
100.0	0.97
200.0	0.98
200 0	0.97

carboxylation by the enzyme system of *Lactobacillus arabinosus* were fumarate, oxaloacetate and pyruvate. The rate of decarboxylation of oxaloacetate was at about the same order as that of malate and the rate of carbon dioxide evolution from pyruvate depended greatly on pyruvate concentration. Fumarate was shown to react at only 0.02 to 0.05 times the rate of malate because of the weak fumarase activity of the bacterium.

To investigate the interference with the assay of carbon dioxide evolved from these acids, 0.1 ml of 0.4 per cent aqueous aniline was added to the sample to remove oxaloacetate, and 0.2 ml of 0.7 per cent hydroxylamine hydrochloride to eliminate pyruvate. Although the rate of decarboxylation of fumarate was too low to affect the results of analyses significantly, bromine water was added to the sample 1 hour before commencing the experiment to convert fumarate to dibromosuccinate.

The addition of these reagents to the samples had no significant effect on carbon dioxide evolution and the results of determination of malate in grape must and wine treated with anailine, hydroxylamine hydrochloride and bromine water are given in table 2

TABLE 2

The Determination of l(—) Malic Acid in Grape Must and Wine in the Presence of Aniline, Hydroxylamine Hydrochloride and Bromine Water at 25°C

	Addition	Malic acid determined (mg in 1 ml sample)
Grape must (Shiraz)	None	1.28
	None	1.26
	0.1 ml 0.4% aniline	1.27
	0.1 ml 0.4% aniline	1.24
	0.2 ml 0.7% hydroxylamine hydrochloride	1.27
	0.2 ml 0.7% hydroxylamine hydrochloride	1.30
	0.5 ml bromine water	1.29
	0.5 ml bromine water	1.30
Grape must (Riesling)	None	1.14
	None	1.16
	0.1 ml 0.4% aniline	1.14
	0.1 ml 0.4% aniline	1.15
	0.2 ml 0.7% hydroxylamine hydrochloride	1.16
	0.2 ml 0.7% hydroxylamine hydrochloride	1.15
	0.5 ml bromine water	1.16
	0.5 ml bromine water	1.17
Wine (Shiraz)	None	1.68
	None	1.66
	0.1 ml 0.4% aniline	1.64
	0.1 ml 0.4% aniline	1.69
	0.2 ml 0.7% hydroxylamine hydrochloride	1.70
	0.2 ml 0.7% hydroxylamine hydrochloride	1.65
	0.5 ml bromine water	1.69
	0.5 ml bromine water	1.65
Wine (Riesling)	None	1.48
	None	1.46
	0.1 ml 0.4% aniline	1.46
	0.1 ml 0.4% aniline	1.50
	0.2 ml 0.7% hydroxylamine hydrochloride	1.47
	0.2 ml 0.7% hydroxylamine hydrochloride	1.44
	0.5 ml bromine water	1.48
	0.5 ml bromine water	1.49

Because of the high alcoholic strength of some fortified wines the tolerance of the enzyme system to ethanol had to be tested before the method could be used with confidence for malate determination in wines. The combined results of determination of malate in the presence of an increasing concentration of ethanol over the range 0 to 58.8 per cent are listed in table 3.

TABLE 3

The Determination of l(—) Malic Acid in a Standard Aqueous Solution Containing 1 mg l(—) Malic Acid in the Presence of Ethanol at 25°C

Addition of ethanol (ml)	Ethanol in Warburg cup (%)	Malic acid determined (mg)
None	.....	0.92
None	.....	0.94
0.05	1.5	0.93
0.05	1.5	0.92
0.10	2.9	0.93
0.10	2.9	0.94
0.25	7.4	0.92
0.25	7.4	0.93
0.35	10.3	0.95
0.35	10.3	0.92
0.50	14.7	0.93
0.50	14.7	0.92
0.75	22.1	0.93
0.75	22.1	0.92
0.80	23.5	0.92
0.80	23.5	0.97
0.85	25.0	0.59
0.85	25.0	0.54
0.90	26.5	0.52
0.90	26.5	0.48
0.95	28.4	0.22
0.95	28.4	0.23
1.00	29.4	0.13
1.00	29.4	0.15
1.10	32.4	0.08
1.10	32.4	0.04
1.50	44.1	0.06
1.50	44.1	0.04
2.00	58.8	0.06
2.00	58.8	0.02

Considering the degree of dilution of the sample in the Warburg cup before analysis, even the strongly fortified wines fall well within the safety limits of the method. The sudden inhibition of the enzyme system by 24 per cent ethanol is the reaction mixture is clearly shown in figure 1 where the results obtained in a typical experiment with 14 manometers are illustrated.

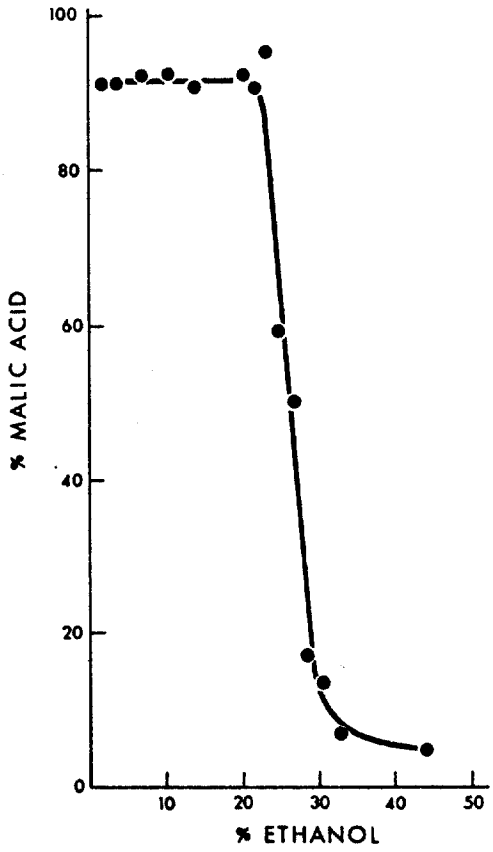

Figure 1. The inhibition of malic decarboxylase by an increasing concentration of ethanol in the determination of l(—) malic acid in a standard aqueous solution containing 1 mg l(—) malic acid at 25°C.

The accuracy of the method was finally established by determining malic acid in samples of grape must and wine which were enriched with incremental additions of l(—) malic acid. The results of these determinations are listed in table 4.

The manometric analytical method gave consistent results with an acuracy of 3 per cent between duplicate and repeated

TABLE 4

The Determination of l(—) Malic Acid in Grape Must and Wine Containing Incremental Addition of l(—) Malic Acid at 25°C

	Malic acid added (mg)	Malic acid determined (mg)
Grape must (Shiraz)	None	1.25
	None	1.29
	0.2	1.47
	0.2	1.43
	0.6	1.81
	0.6	1.79
	1.0	2.20
	1.0	2.16
Grape must (Riesling)	None	1.17
	None	1.14
	0.2	1.33
	0.2	1.31
	0.6	1.69
	0.6	1.72
	1.0	2.08
	1.0	2.12
Wine (Shiraz)	None	1.69
	None	1.63
	0.2	1.87
	0.2	1.81
	0.6	2.20
	0.6	2.16
	1.0	2.57
	1.0	2.54
Wine (Riesling)	None	1.44
	None	1.49
	0.2	1.65
	0.2	1.61
	0.6	2.03
	0.6	1.96
	1.0	2.33
	1.0	2.31

determinations when grape musts and wines were analysed. However, the determinations based on control solution of commercial l(—) malic acid were often consistently low by 8 (±3) per cent of the theoretical values. These low results were explained by chromatographic evidence which showed that all five brands of malic acid tested contained an acidic residue that was left on the starting line of the chromatograms. Such residues, however, were absent from chromatograms run on grape musts and wines. After rigorous purification of the reference compound by preparative paper chromatography, the determination from controls improved and came within 3 per cent of the theoretical values.

SUMMARY

The manometric determination of malic acid is specific, rapid and more convenient than other methods of analysis and, by introduction of 0.5 to 1 ml sample, it extends well over the whole range of concentrations of malate found in grape musts and wines. No preliminary treatment, such as the removal of tartrate and sugars, is necessary and the sample is directly introduced into the Warburg flask. The method has been tested for additional carbon dioxide evolution from fermentation concomitant with malate decarboxylation and from decarboxylation of oxaloacetate, pyruvate and fumarate and no interference from these side reactions with the analysis was detected.

The effect of ethanol on the enzyme system has been investigated. It was found to have no inhibitory influence on the decarboxylation unless the overall concentration exceeded 24 per cent by volume. The accuracy of the method was confirmed by determining malic acid in samples of grape must and wine containing incremental additions of the pure compound.

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CHROMATOGRAPHIC SEPARATION AND DETERMINATION OF FRUIT ACIDS

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The successful application of salting-out chromatography to the analysis of mixtures of alcohols[1], amines[2], aldehydes[3], ketones[3], and ethers[4] led to an unsuccessful attempt to apply this method to the principal acids of fruit (citric, malic and tartaric). The distribution ratios, C, of these acids with Dowex 50 were too nearly equal to permit a satisfactory separation[4]. When it was found that partly sulfonated polystyrene resins gave better separations than the fully sulfonated resins[5,6], it seemed advisable to try these low-capacity resins in the salting-out chromatography of the fruit acids.

Although the procedure for the separation of these acids by ion-exchange chromatography[7] has served satisfactorily in some laboratories, complaints[8] have been received that the permanganometric method for the analysis of the eluate fractions

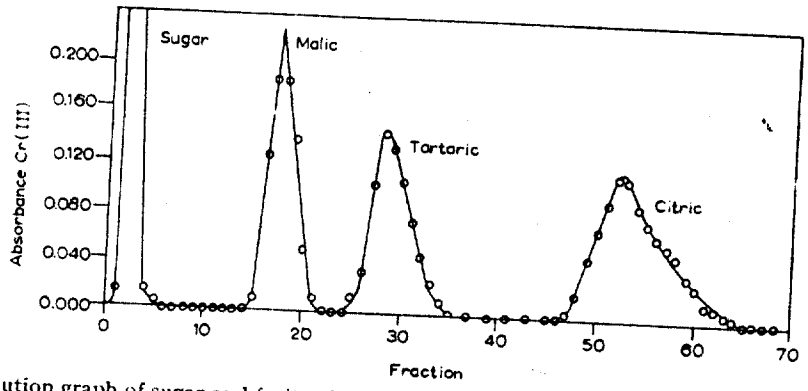

Fig. 1. Elution graph of sugar and fruit acids. Column: 10.0 cm × 0.95 cm² of Dowex 1-X8, 200–400 mesh. Eluent 2.0 M acetic acid + 0.40 M sodium acetate. Flow rate: 0.50 cm/min. Fractions: 2.92 ml.

not reliable. The spectrophotometric method with dichromate[9] has been found to very reliable[1–6] but is not applicable in the presence of nitrate, which was used as the eluent in the separation of the fruit acids by ion-exchange chromatography. These facts furnished an additional incentive for the re-examination of the separation of the fruit acids.

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A method[10] was developed for the quantitative separation by salting-out chromatography of oxalic, tartaric, malic, citric, and lactic acids by elution with a sulfate–bisulfate buffer through a 100-cm column of an incompletely sulfonated polystyrene (capacity = 4.1 mequiv. per g, 4% crosslinked). The method suffered from several disadvantages; the worst of these was a very slow flow rate that required an elution period of five days.

Attention was then turned toward anion-exchange chromatography with various buffer systems. Two fairly rapid methods for the quantitative separation of malic, tartaric and citric acids were developed with Dowex 1-X8 as the stationary phase. In one method, the eluent was 0.15 *M* phosphoric acid plus 2.0 *M* monosodium phosphate. The major disadvantage of this method is that the elution graph of tartaric acid (the first of the fruit acids) overlaps badly the graph of the sugars.

With an eluent of 2.0 *M* acetic acid and 0.40 *M* sodium acetate, quantitative separation of the three fruit acids from each other and from the sugars was achieved (Fig. 1). Since acetic acid is much more resistant to oxidation than the fruit acids, it was possible to determine the latter in the eluate fractions by a slight modification of the dichromate procedure[9] without any interference from the acetic acid. The details of this method are given in the next section. Variations in the composition of the acetate eluent affected the positions of the peaks of the fruit acids in accordance with the equations previously published[11].

EXPERIMENTAL

Apparatus and reagents

Prepare the eluent solution to be 0.40 *M* with sodium acetate and 2.0 *M* with acetic acid. The pH should be 4.0 ± 0.1. Prepare the solution of sodium dichromate in concentrated sulfuric acid as described elsewhere[9].

Slurry Dowex 1-X8, 200–400 mesh, with water. Let it settle about 5 min; then pour off the supernatant suspension of the very fine particles. Repeat this procedure several times until most of the fines are removed. Pour a slurry of the resin into a glass tube, 0.95 cm^2 in internal cross-sectional area; provided with a sintered-glass filter disk of medium porosity and a stopcock or pinchclamp. The height of the resin column should now be a little more than 10.0 cm. Pass the eluent through the column until the resin is completely in the acetate form (negative test for chloride in the effluent). If necessary, adjust the height of resin to 10.0 cm.

Preparation of fruit juices

Cut the fruit into suitable pieces and put it on a filter mat of 6 or 8 layers of surgical gauze on a Buchner funnel. Apply vacuum and press the fruit with a Petri dish. Centrifuge the filtrate to remove finely divided solids and store the supernatant juice in a refrigerator until it is to be used.

Pipet a suitable volume of the juice into a small beaker and add 5 *M* sodium hydroxide to a pH of 4.0, measured with a pH meter. Transfer to a volumetric flask and dilute to the mark with eluent. The pipet and flask should be chosen so that the concentration of the most abundant acid is between 4 and 9 mg per ml.

Elution

Drain the surplus eluent from the column until the liquid level is about 1 mm above

the top of the resin. Pipet 1.000 ml of sample solution into the column, taking care not to agitate the resin. Drain the liquid again to within 1 mm of the resin. Rinse the inside wall of the chromatographic tube with about 1 ml of eluent and again drain the liquid almost to the resin. Repeat this rinsing and draining twice more. Connect a supply of eluent to the top of the chromatographic column with an air-tight stopper. Maintain a flow rate of 0.50 ± 0.10 cm per min (0.47 ± 0.09 ml per min) by adjusting the hydrostatic head or the stopcock.

It is then necessary to collect successive fractions of 37.5, 30.0, 50.0, 12.5 and 75.0 ml, which will contain respectively the sugars, malic acid, tartaric acid, waste and citric acid. This is done conveniently as follows: Collect the sugar in a 50-ml volumetric flask to which 12.5 ml of eluent has been added from a buret. Collect the malic acid similarly but add 20.0 ml of eluent to the flask. Use a dry 50-ml flask for the tartaric acid, a 25-ml flask containing 12.5 ml of eluent for the waste and a 100-ml flask containing 25.0 ml of water for the citric acid.

Spectrophotometric determinations

Shake the volumetric flasks containing the sugars, malic acid and tartaric acid. Pipet 20.00 ml of each solution into separate 100-ml flasks. Pipet 5.00 ml of water and 20.00 ml of dichromate in sulfuric acid into each flask. Heat them in a bath of boiling water for 30 min. Then cool them to room temperature in a cold-water bath. Read the absorbances at 591 mμ in 100-mm cells, using as a reference a solution prepared by treating 20.00 ml of eluent in exactly the same manner. A Beckman DU spectrophotometer is recommended.

Determine the citric acid similarly but use 25.00 ml of the eluate fraction, no water and 20.00 ml of the dichromate solution.

Convert the absorbance to mg of sugar or acid from calibration graphs prepared as follows: Prepare a solution of 200.0 mg of malic acid in 100.0 ml of eluent. Pipet aliquots of 1.000, 2.000, 3.000 and 4.000 ml into separate 50-ml volumetric flasks and dilute to the mark with eluent. Treat 20.00 ml of each of these solutions exactly as described above for the fraction of eluate containing the malic acid. Prepare calibration graphs for the sugar (using dextrose) and for the tartaric acid in the same manner. Prepare a standard solution of citric acid containing 200.0 mg in 100.0 ml of eluent. Pipet aliquots of 2.000, 4.000, 6.000 and 8.000 ml into separate 100-ml volumetric flasks. Add 25.00 ml of water to each flask and dilute to the mark with eluent. Treat 25.00 ml of each of these solutions exactly as described above for the fraction of eluate containing the citric acid. All the graphs of absorbance *vs.* concentration of acid or dextrose were linear within the specified range.

Since the nature of the sugars differs from fruit to fruit, the foregoing procedure may not determine the actual concentration of sugar (mg/ml) in the juice but rather yields the concentration of dextrose equivalent to the total fruit sugar in reducing capacity according to the recommended method. Although no experimental comparisons of the reducing capacities of the various sugars were performed, it is likely that the several sugars found in fruit have very nearly the same reducing capacity per g[9].

RESULTS AND DISCUSSION

The analytical procedure was first tested with five standard solutions prepared by

weighing the pure solutes. These results are presented in Table I. The mean recovery of all 11 determinations was 100.3%, indicating good accuracy. The standard deviation of the recovery was 1.0%, indicating good precision.

Then four samples of fruit juice were analyzed in duplicate. In some of the determinations, known amounts of fruit acid were added as indicated in Table II. The

TABLE I

ANALYSIS OF STANDARD SOLUTIONS

Soln. No.	Dextrose		Malic acid		Tartaric acid		Citric acid	
	Taken mg	Recovery %	Taken mg	Recovery %	Taken mg	Recovery %	Taken mg	Recovery %
1	2.48	100.4					8.04	100.0
2	2.67	100.0						
3	25.5	97.6	8.68	101.0				
4	103.2	100.7			9.86	100.2		
5	101.1	100.1	9.31	101.3	6.09	101.5	6.32	100.6

TABLE II

ANALYSIS OF FRUIT JUICES

(The results are expressed as mg/ml)

Fruit	Sugar		Malic acid		Tartaric acid		Citric acid	
	Added	Found	Added	Found	Added	Found	Added	Found
Lemon	0.0	17.7	0.0	3.9	0.0	0.0	0.0	51.7
	0.0	17.2	82.5	84.5[a]	79.7	78.5	0.0	52.8
Mean		17.5						52.2
Apple	0	208	0.00	5.53	0.00	0.00	0.00	0.00
	0	207	0.00	5.60	6.40	6.56	6.71	6.60
Mean		208		5.57				
Peach	0	124	0.00	5.89	0.00	0.00	0.00	4.14
	0	118	0.00	5.87	7.01	7.03	0.00	4.33
Mean		121		5.88				4.24
Seedless grape	0	144	0.00	5.13	0.00	7.22	0.00	0.00
	0	143	0.00	5.00	0.00	6.98	6.78	6.72
Mean		144		5.06		7.10		

[a] This figure represents the quantity originally in the juice plus the quantity added.

average difference between the duplicates was 2 mg per ml for sugar, 0.09 for malic acid exclusive of the lemon juice, 0.22 for tartaric acid and 0.6 for citric acid. When tartaric acid was added to a juice containing none of this acid, the mean recoveries were 100.4% with a standard deviation of 1.9%. The similar figures for the recovery of citric acid were 98.8% and 0.4%. These figures confirm satisfactorily the accuracy and precision predicted from the analyses of standard solutions.

The occurrence in a fruit of an acid other than malic, tartaric and citric would not be detected by this method unless the elution peak happened to occur between the peaks of Fig. 1. Thus the unsuspected acid would cause a positive error in the deter-

n Table I. The mean recovery
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plicate. In some of the deter-
s indicated in Table II. The

ss

acid	Citric acid	
ecovery %	Taken mg	Recovery %
	8.04	100.0
100.2		
101.5	6.32	100.6

ml)

acid	Citric acid	
Found	Added	Found
0.0	0.0	51.7
78.5	0.0	52.8
		52.2
0.0	0.00	0.00
6.5	5.71	6.60
0.00	0.00	4.14
7.03	0.00	4.33
		4.24
7.22	0.00	0.00
6.98	6.78	6.72
7.10		

is the quantity added.

er ml for sugar, 0.09 for malic
and 0.6 for citric acid. When
this acid, the mean recoveries
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mination of malic, tartaric or citric acid. However, this is not a serious disadvantage because significant quantities of acids other than these three are rarely found in fruit.

The elution requires 7 h, about the same as SCHENKER's[7] elution. The simplicity and reliability of the spectrophotometric method of analyzing the fractions of eluate are marked advantages of this method in comparison with SCHENKER's. In addition, the recommended method serves to determine the sugar in the juice. This method is much more rapid and accurate than the classical procedures such as the pentabromoacetone method[12] for the determination of citric acid.

Since ion-exchange resins vary appreciably from batch to batch, it cannot be guaranteed that strict adherence to the recommended elution procedure will always yield quantitative separations of the fruit acids. An analyst using a different batch of Dowex 1-X8 may have to make minor changes in the elution conditions such as the column height or the volumes of eluate containing the isolated acids.

SUMMARY

A method is described for the determination of malic, tartaric and citric acids and sugar in fruit juices. It consists of the separation of these constituents by ion-exchange chromatography through a column of Dowex 1-X8 with an acetate buffer as eluent, treatment of aliquots of the separated constituents with dichromate and sulfuric acid and measurement of the absorbance of the resultant green chromium(III).

RÉSUMÉ

Une méthode est décrite pour le dosage des acides malique, tartrique, citrique et du sucre dans les jus de fruits. La séparation s'effectue par chromatographie au moyen d'échangeur d'ions (Dowex 1-X8) et l'analyse par spectrophotométrie, après traitement au dichromate et à l'acide sulfurique.

ZUSAMMENFASSUNG

Beschreibung einer Methode zur Bestimmung von Äpfel-, Wein -und Zitronensäure sowie des Zuckergehaltes von Fruchtsäften. Die Trennung erfolgt durch Ionenaustauscher-Chromatographie. Die Eluate werden mit Bichromat–Schwefelsäure behandelt und die Absorption der entstandenen grünen Chrom(III)-lösung gemessen.

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Detection of Organic Acids in Fruit Juices by Paper Chromatography

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SUMMARY

Organic acids of fruit juices form individual chromatographic patterns that can serve for identification of the juices as well as for detection of adulteration.

INTRODUCTION

Juices of various fruits are differentiated by their flavor as well as acid and sugar content. This work deals with paper chromatographic acid patterns of fruit juices to determine whether such patterns are characteristic of the fruit. During this investigation we examined organic acid patterns of straight and blended fruit juices.

METHODS

Isolation of organic acids of fruit juices. *Lead acetate method* (Way, 1957). Acids in 10 ml fruit juices were precipitated by addition of an 8% solution of neutral lead acetate. The resulting insoluble lead salts were centrifuged and washed with alcohol. These salts were then treated with hydrogen sulfide to convert them into insoluble lead sulfide and soluble free organic acids. The acids were separated by filtration, and the aqueous acid solution was evaporated to dryness. The residue was then redissolved in 1 ml of deionized water. This solution was used for spotting on no. 1 Whatman paper.

Anion exchange method (Anon., 1959; Sullivan *et al.*, 1960). Dow anion exchange resin 1-X10 was converted from the chloride to the formate form by washing with 1*M* sodium formate solution. Twenty ml of single-strength fruit juice were passed through a column packed with the above resin. The resin was washed with water to remove all other juice solids such as color and sugar. The fruit acids were eluted by passing 6*M* formic acid through the column. The eluate containing the organic acids was evaporated to dryness to remove formic acid, and the residue was taken up with 1 ml 35% alcohol. The resulting solution was used for spotting on 12×12-in. Whatman paper.

Solvent systems and development of chromatograms. Two systems were used:

1) Butanol, 90% formic acid, water (10:2:5) was employed for descending chromatography. Nine lambda volume of acid solution was deposited on 12×12-in. strips of no. 1 Whatman paper by multiple spotting (three 3-lambdas.) The solvent was allowed to descend 9 in. from the spotting line, which took about 4 hr at room temperature (70°F). The chromatograms were dried 12 hr to remove volatile formic acid. Acid patterns were made visible by application of indicators.

2) Ether, 90% formic acid, water (60:14:9) was used for ascending chromatography. Nine lambdas were deposited by multiple spotting (three 3-lambdas) on no. 1 Whatman paper 7×16-in. strips. The solvent was allowed to ascend 9 in. from the spotting line, which took 6 hr at 70°F. Chromatograms were dried as indicated above. A mixture of indicators (Fitelson, 1961; Paskova and Mund 1960; Hartly and Lawson, 1960) was applied after drying by dipping or spraying. This mixture contains 0.075% bromcresol green, 0.025% bromphenol blue, and 0.01% 1,3-di(*o*-tolyl) guanidine in anhydrous methanol.

Samples of fruit juices. The following fruit juices and varieties thereof were examined: red raspberry (Newburgh), black raspberry (Munger-Olum farmer blend), red cherry (Montmorency), blackberry (Evergreen), strawberry (Marshall), grape (Concord), apple (variety unknown), and blends of 30% apple and 70% black raspberry and of 30% grape and 70% black raspberry. Organic acids of these juices were isolated by procedures mentioned above. For comparison and identification we prepared solutions of acids known to occur predominantly in plants and juices.

RESULTS

Chromatographic patterns and R_f values. Table 1 gives R_f values and indicates the intensity of chromatographic spots.

Interpretation of chromatograms. The following deductions were made by comparing R_f values of known acids with R_f values of acids isolated from juices:

Strawberry juice. Main acid constituents are citric acid and an unknown acid (R_f 0.75), somewhat less

Table 1. R_f values and relative spot intensities* of known organic acids vs. organic acids in fruit juices and juice blends.

	.91–.94	.79–.85	.72–.75	.60–.64	.51–.56	.43–.44	.32	.19–.28	.11	.07–.08
Malic Acid					.56					
Citric Acid						.44				
Tartaric							.32			
Malonic		.81								
Galacturonic										
Gluconic										.08
									.11	
Blackberry	.94 D	.85 D		.64 F	.56 D	.45 F				.08 D
Red Cherry		.85 VI		.63 VF	.54 VI			.23 VF		.08 VF
Strawberry	.93 D	.86 I			.53 I	.44 I		.21 VF		.08 F
Red Raspberry			.73 VI	.60 VF	.51 VF	.43 VI				.07 F
Grape	.91 I		.72 D	.64 F	.54 I		.32 I			0.83 VF
30% Grape 70% Bl. Raspberry	.93 D	.83 I	.72 VF	.64 VF	.55 F	.45 I	.32 F			.083 F
Bl. Raspberry		.79 VI		.62 VF	.53 VF	.44 VI		.22 VF		.08 F
30% Apple 70% Bl. Raspberry	.93 D	.82 I		.63 VF	.53 D	.42 D		.20 VF		.07 F
Apple	.90 D	.82 I			.53 VI			.19 F		.07 VF

* VI, very intense; I, intense; D, distinct; F, faint; VF, very faint.

malic acid, little galacturonic acid, and three additional acids of R_f values 0.93, 0.86, and 0.23.

Black raspberry juice. Six acids are present. Predominating are citric acid and an unknown acid (of R_f 0.79); malic acid and galacturonic acids are present in small quantities; the other acids were not identified.

Red raspberry juice. The major acid is citric acid; also present are very little malic, galacturonic, and two other minor acids.

Grape juice. Malic and tartaric acids are predominant; citric, galacturonic, and three other acids are present in lesser quantities.

Apple juice. The major acid is malic acid; four other acids are found.

Blackberry juice. Contains a combination of six acids, including citric, malic, and galacturonic, but none predominate.

Red cherry juice. Contains mainly malic acid. Four additional acids are present.

Pineapple juice. Citric acid is the main acid. Malic and tartaric acids are minor acid constituents.

Prune juice. Main acid constituents appear to be galacturonic and pectinic acids; small quantities of tartaric and citric acids are present.

Blended juices. The acid chromatograms of blended juices provide a clue to their composition. An acid pattern can also indicate adulteration if foreign acids are detected. The acid pattern in black raspberry juice is changed when apple juice or grape juice is added. In the first case we notice great intensification of the malic acid spot, whereas blend with grape juice shows presence of tartaric acid, which is foreign to black raspberry juice.

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Yeast Growth as Affected by Sodium Benzoate, Potassium Sorbate and Vitamin K5

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SUMMARY

Five selected yeast strains were grown in a standard apple juice-sucrose medium containing potassium sorbate, sodium benzoate, and vitamin K5. Changes in the refractometer readings of the medium were used as a criterion of yeast growth. This method of assessing yeast growth is subject to a number of interfering factors. However, for comparative purposes these are not considered critical. Potassium sorbate exerted a greater inhibiting effect than did sodium benzoate, throughout a range of pH 6.0–4.0 and a range of concentration of 0.025–0.1%. The action of both benzoate and sorbate was enhanced by lowered pH. Vitamin K5 showed no suppressing action (with one exception) on the growth of the selected strains of yeast, in concentrations of 0.0025–0.01% throughout the pH range 6.0–4.0.

INTRODUCTION AND LITERATURE REVIEW

Sodium benzoate, used as a preservative of food products for many years, is still preferred for fruit products and pickles. It has several disadvantages, however: it is difficult to dissolve, it imparts an undesirable flavor to many foods (Dryden and Hills, 1959; Weaver *et al.*, 1957), and it is not handled in the body by normal metabolic pathways (Deuel *et al.*, 1954a,b).

Potassium sorbate, in contrast, possesses the distinct advantages of being readily metabolized (Deuel *et al.*, 1954a,b) and easily soluble, and contributing no undesirable flavor when used in effective concentrations (Dryden and Hills, 1959; Weaver *et al.*, 1957).

This paper presents data comparing the activity of these compounds, as an introductory phase of a study on the use of potassium sorbate in the preservation of fresh apple juice.

Vitamin K5, reported as preventing secondary fermentation in wines (Yang *et al.*, 1958), was included in this study because of its possible use in preservation of fresh apple juice or fermented fruit beverages.

EXPERIMENTAL METHODS

The inhibitory effect of potassium sorbate (Union Carbide Chemicals Co., New York, N. Y.), sodium benzoate (J. T. Baker Chemical Co., Phillipsburg, N. J.), and vitamin K5 (2-methyl-4-amino-1-naphthol hydrochloride) (Nutritional Biochemicals Corp., Cleveland, Ohio) was evaluated by growing pure cultures of selected yeasts in an apple juice-sucrose medium containing the substance to be tested, and measuring the reduction in total soluble solids content of the medium. Determinations of total soluble solids were made over a 21-day period with an Abbe-type refractometer.

Changes in refractometer reading of the medium during an experiment reflect metabolic activity rather than the absolute growth of the organism. There is a rough correlation between metabolic activity and growth; however, since this paper is concerned with compounds that are designed to prevent spoilage, i.e., changes in the medium, the more direct method of measuring the ability of the organism to change its environment seemed better than measurement of the number of organisms alive or dead.

Basal medium. The basal medium was prepared according to the following formula: 500 ml apple juice, 500 ml distilled water, 3 g dipotassium phosphate, 1 g ammonium chloride. Sucrose was added to give a total soluble solids content of 23% as estimated by refractometer.

The apple "juice" used was prepared from McIntosh apple juice concentrate of 76% total soluble solids. For use in the medium the concentrate was reconstituted with distilled water to a total soluble solids content of 12%. This procedure produced a

Determination of Fixed Acids in Commercial Wines by Gas–Liquid Chromatography

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The polybasic acids in wines (succinic, fumaric, malic, tartaric, and citric) were separated from other solid material by precipitating the acids as the lead salts of the respective acids. Trimethylsilyl derivatives were formed directly from the precipitates and were identified and quantitated by vapor phase chromatography. It is interesting to note that blackberry, peach, pineapple, elderberry, loganberry, honey, and cherry wines did not contain tartaric acid in detectable amounts (10 mg/100 cm^3 of sample). The citric acid concentration in commercial wines in some cases accounted for approximately 90% of the total fixed acid content. Grape wine in all cases contained malic and tartaric acids and in most cases contained citric acid.

Customarily the separations of carboxylic acids by glc are preceded by the conversion of the acids to alkyl (usually methyl) esters. Since the esters are more volatile, they can be separated at lower temperatures. These esters are less strongly adsorbed than acids and give less tailing; therefore, they can be more accurately determined.

Phenols (Ismail, 1963; Langer *et al.*, 1958) were the first compounds to be separated by gas chromatography as their silyl derivatives. Silyl ester derivatives have been used for identification of the anomers of pentoses and hexoses by gas chromatography (Bentley *et al.*, 1963; Sweeley *et al.*, 1963; Martin and Eib, 1968). Flavenoids (Furuya, 1964) and inositols (Lee and Ballou, 1965) have also been converted to silyl derivatives for gas chromatography. The silyl ether derivatives of large complex molecules such as morphine (Brochmann-Hanssen and Svendsen, 1963; Martin and Swinehart, 1966) and steroids (Hammond and Leach, 1965) and mono- and diglycerides (Tallent *et al.*, 1966; Wood *et al.*, 1963) have been chromatographed. The use of silyl esters is a logical extension of this work to gas partition chromatography and the gas chromatography of trimethylsilyl esters of fatty acids (Birkofer and Donike, 1967; Dalgliesh *et al.*, 1967), amino acids (Mason and Smith, 1966; Ruhlmann and Giesecke, 1961), metabolic acids (Horii *et al.*, 1965), acids in wine (Brunelle *et al.*, 1967), acids in whiskey (Martin *et al.*, 1965), phenolic acids (Burkhard, 1957; Blakley, 1966), resin acids (Zinkel *et al.*, 1968), and fruit acids (Fernandez-Flores *et al.*, 1970).

The various reagents that are used to synthesize the alkyl esters do not react with alcohol groups such as those on hydroxyl acids. If, however, hydroxycarboxylic acids are combined with trimethylchlorosilane, both the acid and alcohol hydroxy groups react to give the trimethyl siloxy group. Therefore, the trimethylsilyl derivatives of hydroxycarboxylic acids are more volatile and less easily adsorbed than alkyl esters of hydroxycarboxylic acids. The silyl ether esters are superior to the methyl esters for gas chromatographic separations of hydroxycarboxylic acids (Martin and Swinehart, 1968).

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METHOD

Apparatus and Reagents. GAS CHROMATOGRAPH. A Hewlett-Packard Model 402 gas chromatograph equipped with a flame ionization detector was used for the analysis. This instrument was placed in tandem with a Hewlett-Packard Integrator Model 3370A. The glass column for the instrument was cleaned with acetone and ethanol, and then allowed to air dry. A 250-ml solution of a 5% dimethyldichlorosilane (DMCS) in benzene was passed through the column, after which it was allowed to air dry.

The glass column for the analysis was 6-ft long, 1/4-in. o.d., and was packed with a 3% OV-3 on Chromosorb W HP 80–100 mesh. The glass wool plugs were likewise treated with 5% DMCS in benzene.

The column was operated isothermally for 10 min at 118° C and then programmed at the rate of 3°/min to a temperature of 220° C. The detector block and injection port were operated at 250° C. Helium was used as the carrier gas at the rate of 40 ml/min.

Lead acetate buffer solution 8 g $Pb(OAc)_2 \cdot 3\ H_2O$ plus 1 ml of acetic acid, and the final solution *qs* to 100 ml with water.

Celite. Celite 545 (Fisher Chemical).

Sulfuric acid. 0.1 *N* sulfuric acid.

Glutaric acid. A 1-mg/ml solution of glutaric acid (Applied Science Laboratories, Inc., 99.9% purity) in a 50/50 ethanol water solution.

Standard acid solution. A 5×10^{-2} *M* solution each of succinic acid (Fisher Certified), fumaric acid (Eastman Organic Chemicals) 98% + purity, l malic acid (Eastman Organic Chemicals) 98% + purity, tartaric acid (Eastman Organic Chemicals) 98% + purity, and citric acid (Fisher Scientific) anhydrous certified grade were placed in a 50/50 ethanol water solution.

Table I. Total Acid Values by Titrimetric Method, Individual Acids by the Glc Procedure

Name	Gas Liquid Chromatography					Titrimetric		
	Milliequivalent				Mg/100 cc.	Mg/100 cc.		
	Succinic Acid	Malic Acid	Tartaric Acid	Citric Acid	Total Fixed Acid*	Fixed Acid*	Volatile Acid*	Total Acids*
Apple Wine	1.253	6.205		0.484	596.0	543.0	47.0	590.0
Blackberry Wine	0.627	1.193		4.950	508.0	579.0	81.0	660.0
Blackberry Wine	0.881	2.685		3.826	555.0	642.0	128.0	770.0
Chianti Wine	1.626	1.462	2.692	0.437	467.0	554.0	76.0	630.0
Concord Grape Wine	1.135	0.746	2.999		366.0	480.0	130.0	610.0
Danish Blackberry Wine	0.406	0.985	0.133	2.077	270.0	295.0	232.0	527.0
Danish Cherry Wine	0.745	6.862	1.346	0.250	691.0	673.0	66.0	739.0
Dry Red Wine I (Before Treatment)	0.932	0.149	4.544	0.281	518.0	533.0	108.0	641.0
Dry Red Wine II (Before Treatment)	0.982	0.149	4.598	0.281	451.0	545.0	96.0	641.0
Dry Red Wine III (Before Treatment)	1.745	Trace	3.505	0.531	434.0	580.0	134.0	714.0
Dry Red Wine IV (After Treatment)	1.423	Trace	3.691	0.562	426.0	557.2	166.8	724.0
Elderberry Wine	0.999	0.567		5.075	498.0	576.0	104.0	680.0
Grape Base Wine	0.999	0.627	2.559	Trace	314.0	341.0	71.0	412.0
Loganberry Wine	0.627	1.119		9.072	812.0	612.0	98.0	710.0
Muscatel Wine	0.881	1.298	1.626	1.499	398.0	296.0	74.0	370.0
Peach Wine		0.269		2.171	183.0	193.0	186.0	379.0
Polish Cherry Wine	1.135	1.566		5.434	610.0	658.0	150.0	808.0
Port Wine	0.406	1.477	2.132	0.312	325.0	332.0	108.0	440.0
Pure Cherry	0.745	3.058		2.030	438.0	508.0	92.0	600.0
Natural Grape Wine Base	0.999	0.507	3.252	0.484	393.0	377.0	110.0	487.0
Red Dry Wine	1.507	2.506	5.735		732.0	672.0	98.0	770.0
Straight Sherry Wine	1.762	0.627	2.132	0.968	412.0	304.0	86.0	390.0

* Expressed as Tartaric Acid

Pyridine. Pyridine (Eastman Organic Chemicals) spectrograde was dried for 48 hr over KOH granules.

Silylating reagent. Trimethylchlorosilane (TMS) and hexamethyldisilazane (HMS) were obtained from Applied Science Laboratories, Inc.

Ethanol water solution. 80% anhydrous U.S.P. and 20% distilled water.

Sample Preparation. A 2-ml aliquot of wine was placed in a 15-ml centrifuge tube, and 1 ml of lead acetate buffer solution, 10 mg of celite 545, and 1 ml of sulfuric acid were added in that sequence. The precipitate was shaken with 10 ml of ethanol water solution, after which it was centrifuged for 10 min. The tube was removed and the supernatant decanted. The mat was broken with a stirring rod, and a second 10-ml

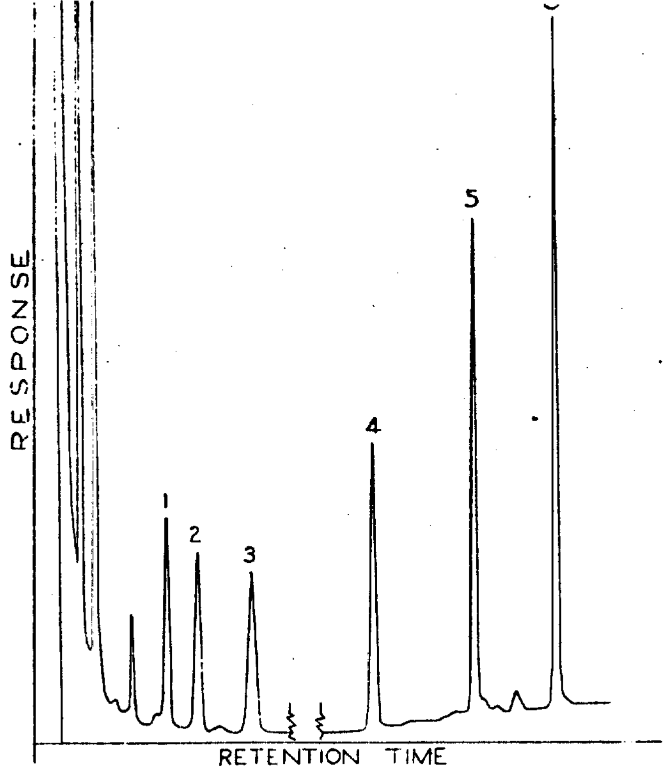

Figure 1. Chromatogram of a standard solution of acids: 1, succinic; 2, fumaric; 3, glutaric; 4, malic; 5, tartaric; 6, citric

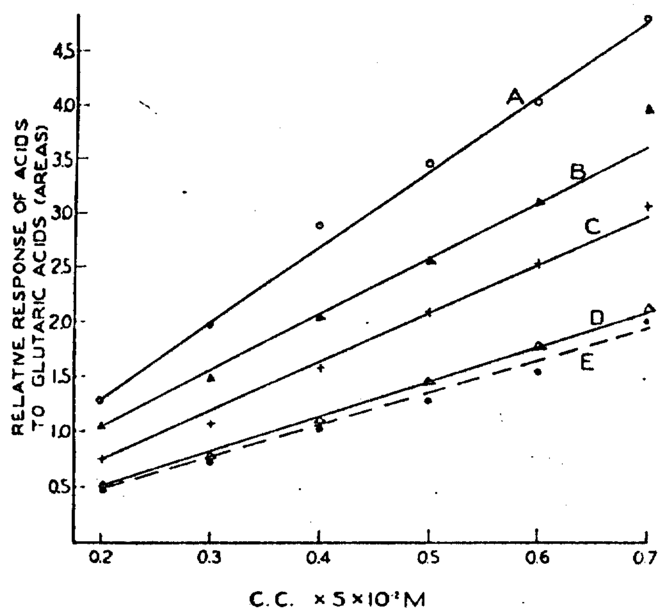

Figure 2. This gives the relative response of A, citric, B, malic, C, tartaric, D, succinic, and E, fumaric, to glutaric acids *vs.* cm³ molar concentration

ethanol–water solution was employed to wash the precipitate. The mixture was centrifuged for another 10 min, and again the supernatant was decanted. Boiling chips were added, along with 1.5 ml of glutaric acid solution, to the precipitate, and the mixture was placed in an oven for 12 hr at 105° C. Pyridine, 1.5 ml, was added to the mixture, which immediately was capped with a rubber septum and then placed in a sand bath for 24 hr at 137° C. The sample was removed from sand bath, and 0.5 ml of TMS and 0.5 ml of HMS were added. The resulting solution was allowed to stand at room temperature graphic analysis.

Standard Solution. 0.2, 0.3, 0.4, 0.5, 0.6, and/or 0.7 ml of a 5×10^{-2} *M* solution of succinic, fumaric, malic, tartaric, and citric acids were placed in a 15-ml centrifuged tube, respectively. The standard was treated in the same manner as the sample solution.

Calculation. The amount of organic acids was calculated in the following manner:

$$\frac{Ao/Gs}{Astd/Gstd} \times Mg \times 50 = mg/100\ cm^3 \text{ of sample}$$

where Ao = area of organic acid, Gs is the area of glutaric acid in sample, Astd is the area of organic acid in standard solution, and Gstd is the area of glutaric acid in standard solution. A blank sample (before introduction of internal standard) was chromatographed to ascertain possible interference from the presence of glutaric acid or other compounds with similar retention times. The recovery of fixed acids by the glc procedure was compared with recovery by titrimetric methods. The amount of fixed acids found by titrimetric methods was determined as the difference between total acids and volatile acids.

The procedural modifications employed were as follows. The end point was determined by a Fisher Automatic Titrimeter at pH 8.4 instead of the visual point of phenolphthalein.

DISCUSSION

The di- and tricarboxylic acids used in the determination were succinic, fumaric, glutaric, malic, tartaric, and citric. The retention times of the various acids for the standard solution were noted and compared with those of the sample solution.

In prior work, an alkanoic acid (C_{11}) was used as an internal standard, but because of its high degree of sublimation and its monocarboxylic character, it necessitated the use of dicarboxylic or tricarboxylic acid not present in wines. Since glutaric acid was not found to occur naturally in any of the samples analyzed by glc it was selected as the internal standard of choice. The various acids were determined in wines, but further studies would be highly desirable as to varieties within a particular type of wine, *i.e.*, catawba grapes wine. Imported and domestic commercial samples were selected in order to provide a fair degree of diversification.

RESULTS

Figure 1 shows the various acids, namely succinic, fumaric, glutaric, malic, tartaric, and citric, as sharp symmetrical peaks 1, 2, 3, 4, 5, and 6, respectively.

Figure 2 gives linearity of response of molar concentration of acid to glutaric acid by peak areas. The acids fall into a sequence which is proportional to their molecular weights.

Table I gives data for blackberry, cherry, elderberry, apple, loganberry, peach, and various classes of grape wines. In many cases, the citric acid content accounts for 90% of the fixed acid content for the commercial wines. As was expected, the malic acid is found in apples, pineapple, blackberry, loganberry, cherry, grape, elderberry, and peach wines. Tartaric acid is found in wines of grape origin, but it is not found in apple, blackberry, elderberry, loganberry, cherry, or peach wines at detectable levels (10 mg/100 ml).

In most cases, the fixed acid values found by glc procedures are lower than titrimetric values.

Samples of Dry Red Wine I and II, as well as III and IV, show before and after cation exchange treatment. For this particular treatment, there appears to be no significant change in the malic, succinic, citric, or tartaric concentration.

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Quantification of Seven Tricarboxylic Acid Cycle and Related Acids in Human Urine by Gas–Liquid Chromatography

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Biologically significant concentrations of pyruvate, lactate, fumarate, succinate, malate, alpha-ketoglutarate, and citrate in human urine were quantified by gas–liquid chromatography. The urine was first deproteinized, neutralized, and passed through an ion exchange column. The resulting acids were esterified. The esters were separated on temperature programmed 12.5% diethyleneglycol succinate and 6% XE-60 nitrile rubber columns and detected by hydrogen flame ionization. Unknown concentrations were calculated from individual regression equations, derived from the response of either the commercial ester or the esterified acid. In general, with a 5.9-μl injection, the responses were linear at least in the range of 1.26 to 25 μmoles/ml. Mean recoveries depended on the individual acid or salt and varied from 76.4 to 127% with some exceptions. Recoveries from urine varied similarly. Five replicate analyses resulted in relative standard deviations of 4.2 to 25.9% Neither esterified oxaloacetate nor isocitrate yielded a characteristic peak; esterified oxalosuccinate and cis-aconitate gave multiple peaks.

THE SEQUENTIAL conversion of carboxylic acids into CO_2 and H_2O is a characteristic feature of cell metabolism. The turnover of these acids, described by Krebs (*1*), is known as the tricarboxylic acid (TCA) cycle and represents a common pathway for metabolism of carbohydrates, fatty acids, and amino acids. The levels of these acids in a tissue during a steady state is an index of the activity of the pathway in the cells. The kidney is an important regulatory organ which depends on TCA cycle metabolism for many of its functions. There is some evidence that disturbances of kidney function by disease or by metabolic disorders may be reflected by abnormal patterns of excretion of the TCA cycle metabolites, indicating either some distortion in their synthesis or in their utilization by kidney cells (*2*). Although plausible, this hypothesis has been difficult to test because of methodological problems for quantifying TCA cycle acids in urine. Enzymatic methods for individual metabolites are available (*3*) but are expensive, and several metabolites cannot be quantified from a single reaction. Chemical methods are adequate only for a few of the TCA cycle related metabolites. Various chromatographic methods other than gas chromatography have been developed as an alternative to these (*2, 4–7*).

The advantages of gas chromatography as a method of analysis are the ability to determine several compounds in one analysis of a small amount of sample and the possibility of simultaneous radioactivity determination with a radiologic detector. Although the actual gas-chromatographic analysis can be performed in several minutes, the preparation of the sample for analysis and the calculation of the results require many hours. Derivatives of one or more of the TCA cycle acids from sources other than biological fluids have been separated, identified (*8–12*), and measured (*13–18*) by gas chromatography. Some of the acids have been extracted from biological fluids and subsequently separated and identified by this technique (*19*). Quantifications have been infrequent (*20*). Dalgliesh *et al.* (*21*) have described a method for separation and identification of a wide range of metabolites in urine, including some of the quantitative aspects and problems of derivative formation; however, the recoveries of these acids from the several steps of urine preparation were not reported. Kuksis and Prioreschi (*20*), using a method with an ion exchange procedure similar to ours, have reported recoveries of 89 to 113% of some of these acids from urine in amounts 2 to 90 times those used in the present study.

The present paper describes detailed investigations of the quantitative analysis of pyruvate (2-oxopropanoate), lactate (2-hydroxypropanoate), fumarate (transbutenedioate), succinate (butanedioate), malate (hydroxybutanedioate), α-ketoglutarate (2-oxopentanedioate), and citrate (2-hydroxy-1,2,3-propanetricarboxylate) by gas chromatography with special application to urine.

EXPERIMENTAL

Reagents. Reagents of the highest purity available were obtained from various commercial sources: acids, salts (except barium oxalosuccinate) and esters of and related to the TCA cycle acids, isocitric acid lactone, adipic, malonic

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Table I. Operating Conditions

Type of column: 12.5% DEGS on acid washed Chromosorb W (45–60 mesh) and 6% XE-60 on Gas Pack S (45–60 mesh) in 6′ × 4 mm (i.d.) glass tubing.
Gas flows:
  Carrier (Helium):
    60 ml min through each column and
    120 ml min through each burner.
  Hydrogen: 55 ml min through each burner.
  Air: 300 ml min through each burner.
Temperatures:
  Inlet (top of column): 220 °C
  Detector oven: 255 °C
  Column: Programmed: 80 °C for one minute, 80–100 °C at 2.9 °C min, 100–133 °C at 4.7 °C min, 133–220 °C at 4.8 °C min, 220 °C for 2.5 minutes.

Table II. Absolute and Relative Retention Times of Methyl Esters

Methyl esters (in order of elution)	Number of samples	Retention time from solvent front (minutes) Mean ± S.D.	Relative retention (retention time of ester retention time of methyl laurate) Mean ± S.D.
Pyruvate	15	1.86 ± 0.23	0.169 ± 0.019
Lactate	15	2.25 ± 0.24	0.205 ± 0.019
Fumarate	15	7.31 ± 0.30	0.664 ± 0.017
Succinate	15	9.17 ± 0.27	0.835 ± 0.011
Laurate	15	11.00 ± 0.21	1.000
Malate	14	19.91 ± 0.10	1.811 ± 0.032
α-Ketoglutarate	9	21.43 ± 0.10	1.929 ± 0.025
Citrate	16	29.66 ± 0.10	2.698 ± 0.048

and tartaric acids (Sigma Chemical Co., St. Louis, Mo., and Perco Supplies, San Gabriel, Calif.); barium oxalosuccinate (Nutritional Biochemicals Corp., Cleveland, Ohio); fatty acid methyl esters (Applied Science Labs., Inc., State College, Pa.); glycolic and oxalic acids and gas chromatography grade methanol (Fisher Scientific Co., Chicago, Ill.), 10% boron trifluoride-methanol (Eastman Organic Chemicals, Rochester, N. Y.); the anion exchange resin, AG 2-×8, in the formate form, 100–200 mesh, prepared from the corresponding Dowex resin (Bio-Rad Labs., Richmond, Calif.) and the gas chromatographic column packings (Chemical Research Services, Inc., Addison, Ill.).

Gas Chromatography. The instrument used was a Beckman GC-4 with dual columns, including on-column injection and dual hydrogen flame ionization detectors. The signal was recorded with a Beckman linear strip chart recorder operated at the 1-mV range and equipped with a disc integrator. Hydrogen gas was supplied by a Milton Roy Elhygen hydrogen generator. The operating conditions are given in Table I. Identical flows and temperatures were used for both the diethyleneglycol succinate (DEGS) and XE-60 nitrile rubber columns, which were packed in this laboratory and conditioned overnight with a carrier flow of 60 ml/min prior to use. The DEGS column was conditioned at 220 °C; the XE-60 column at 250 °C. The column efficiency of the DEGS columns varied from 7000 to 9000 plates per column, determined from the dimethyl succinate peak immediately after conditioning.

Injections were made with a Hamilton 10-μl syringe in a manner similar to the technique used by Kuksis and Vishwakarma (*13*) for improved accuracy and reproducibility. Methanol was drawn into the syringe to the 1.5-μl mark. This solvent was then drawn into the barrel, leaving an air space below the 1.5-μl mark. Next, the sample was drawn into the syringe up to the 5-μl mark, pulled entirely into the barrel for more accurate reading, and adjusted, if necessary, to 5.9 μl. During insertion the sample was contained in the barrel of the syringe, leaving the needle empty. The solvent acted as a wash during injection. As previously recommended (*8*), an injection of solvent was made between sample injections (approximately 1-μl of methanol was injected in our procedure) to prevent ghosting. Nine consecutive injections of a solution of methyl lactate in methanol were made under the same conditions to check the reproducibility of the injection. The mean peak area and its relative standard deviation were calculated.

Unknown peaks were identified on the two columns by comparison of their relative retentions to the relative retentions of known esters chromatographed on the same day under the same conditions. Relative retentions were determined with respect to methyl laurate, added just before injection. Identifications using data from only one column were considered tentative. The standard deviations were determined for the retention time (from the solvent front) and the relative retention of each ester. The data, obtained on the DEGS column over a period of 5 weeks, are summarized in Table II.

The attenuator was calibrated by deflecting the recorder pen to 100% at an attenuation of 1 with suppression current and noting the deflection at various attenuation settings. When the pen dropped below 10% scale, it was re-adjusted to 100% with suppression. The actual attenuation (A') at each setting was equal to the product of the ratio of full scale (D) to the observed deflection (d) and the known attenuation at full scale (A):

$$A' = \frac{DA}{d}. \qquad (1)$$

The response of the integrator in integrator units per minute was determined at pen deflections of 1% intervals up to 20% scale and 5% intervals from 20% to 100% scale. The response was linear (Pearson's Coefficient of Correlation = 1.000), and the regression equation for per cent scale *vs.* the corresponding integrator units per minute was calculated. This equation was used to calculate the base-line correction.

Methanol solutions, containing either a single pure ester or mixtures of esters at several known concentrations, were chromatographed. Equal volumes were injected, and the peak areas determined for each concentration. The response of the gas-chromatographic system to the methyl esters of pyruvate, lactate, fumarate, succinate, and citrate was characterized by calculating Pearson's Coefficient of Correlation, a regression equation, and the standard error of the estimate for the concentration *vs.* peak area data. Unknown concentrations of methyl esters of these acids were calculated from the peak area of the unknown, using the corresponding regression equation.

Because the pure methyl esters of malate and α-ketoglutarate could not be obtained commercially, the response of the gas chromatographic system to these esters could not be determined. As an alternative, the overall response, including esterification and the gas chromatographic system, was studied. Various amounts of malic or α-ketoglutaric acid were weighed into 10-ml volumetric flasks, esterified according to the HCl-methanol procedure given below, and then made up to volume with methanol. Equal volumes (5.9 μl) of these solutions were chromatographed under the same conditions on the day after esterification; the linear, semi-log, and log-log plots of acid concentration *vs.* peak area of the resulting ester were obtained.

Preparation of Urine. A known volume of urine, between 10 and 20 ml, was pipetted into a 50-ml centrifuge tube containing 5 ml of 30% $HClO_4$ (*21*) and enough distilled water to bring the final volume to about 25 ml. The contents were mixed, allowed to stand at room temperature for 10 minutes and then centrifuged at 1300 × g for 10 minutes. The supernatant was decanted into a clean 50-ml centrifuge

tube and kept in crushed ice. The precipitate was washed with 10 ml of 3% $HClO_4$, and the suspension was centrifuged as above. The wash was added to the original supernatant. Following neutralization to a pH of 5 with 6*N* KOH and standing for 10 minutes in crushed ice, the mixture was centrifuged at 1300 × g for 5 minutes. The supernatant was transferred to the top of a previously prepared anion exchange column. The $KClO_4$ precipitate was washed with 10-ml of ice-cold distilled water; and, after centrifugation (1300 × g for 5 minutes), the wash also was transferred to the top of the anion exchange column.

The column was prepared by suspending 6.0 grams of AG 2-×8 resin in the formate form, 100–200 mesh, in distilled water in a 50-ml beaker. After a 10-minute settling period, the fines were poured off and the slurry was poured into a 0.9-cm (i.d.) glass chromatographic column with a reservoir top, plugged with glass wool just above a Teflon (Du Pont) stopcock at its lower buret tip. The resin adhering to the side of the beaker was carefully washed into the column with a stream of water. The meniscus was adjusted to just above the top of the approximately 11.5-cm resin column. Care was taken to keep the resin always under water.

The anion exchange procedure was a modification of one proposed by Nordmann and Nordmann (*5*), and Nordmann *et al.* (*4*). The column flow was adjusted to 0.9 to 1.1 ml/min. When the meniscus was just above the resin bed, 63 ml of distilled water were added; and the column was slowly swirled to wash down the sides of the reservoir. Then the water was allowed to pass through at the same flow rate. Adhering acids were eluted, also at the same flow rate, into a 200-ml, round-bottomed flask, using 42 ml of 12*N* formic acid. The eluent was evaporated to apparent dryness with a rotary evaporator under reduced pressure (0.2 mm of mercury) at 33 °C, then kept on the evaporator for 1 minute more. (Excessive evaporation was avoided to prevent increased losses of the more volatile acids.) A 0.5-ml portion of methanol was added to the residue, and the final suspension was transferred with a Pasteur-type pipet to a 1-ml volumetric flask. The round-bottomed flask was washed with three 0.25-ml portions of methanol, similarly transferred with the same pipet.

Esterification. Two methods of esterification were used: HCl–methanol in a manner similar to that proposed by Rumsey and Noller (*17*) and BF_3–methanol, originally proposed for esterification of fatty acids by Metcalfe and Schmitz (*22*).

In the former method 50 µl of concentrated, reagent grade hydrochloric acid was added to the methanol solution of the isolated acids prepared above; and, if necessary, the volume was made up to approximately 1 ml with methanol. The stoppered, Parafilm "M"-covered flask was shaken in a water bath at 55 °C for 4 hours (*17*) and refrigerated, until methyl laurate was added, the volume adjusted to exactly 1 ml with methanol, and the contents were chromatographed. The HCl–methanol technique was checked by triplicate esterifications of a 5-ml aliquot, containing the five free acids or salts whose esters were available commercially, with 0.5 ml of concentrated HCl, and enough methanol to make the volume 10 ml (final concentration of each, about 9 µmoles/ml). The day following esterification, 5 µl of methyl laurate were added to each 10-ml flask. The solutions were made up to volume with methanol, and the resulting solutions were analyzed. The ester concentrations obtained were compared to the initial acid or salt concentrations. Three similar solutions were tested for stability at various intervals during a 2-week period by comparing peak areas, corrected for sensitivity changes of the system.

The optimal esterification time, using BF_3–methanol, was determined in a similar manner. The acids or salts (final concentration of each approximately 25 µmoles/ml) were dissolved in 10% BF_3–methanol, eight 5-ml aliquots were transferred to separate 10-ml volumetric flasks, and the flasks were made up to volume with 10% BF_3–methanol. They were then incubated in a shaker bath as above. One flask was removed at each of the following intervals: 15, 30, and 45 minutes and 1, 2, 3, 4, and 5 hours. The flasks then were treated as above, and yields were calculated from the two samples which had the greatest peak areas.

Recoveries. Recoveries were determined by preparing an aqueous solution, containing all seven acids or salts in known concentration (approximately 0.8 µmole/ml of each acid or salt), taking three 10-ml aliquots and treating them as urine samples, including HCl–methanol esterification. Because, as a result of the method, the components of the 10-ml aliquot were brought to a final volume of 1 ml, a final concentration of approximately 8 µmoles/ml of each ester represented 100% recovery. The mean recoveries were calculated.

The effect of other urine components on the recoveries of the anions was determined. The following were prepared in triplicate: 1) 10 ml of urine and 10 ml of acid–salt standard solution (aqueous) in which each anion, added as either the acid or salt, was at a concentration of 0.1 µmole/ml, 2) 10 ml of urine and 10 ml of H_2O, and 3) 10 ml of acid–salt standard solution and 10 ml of H_2O. All samples were subjected to deproteinization, neutralization, and centrifugation procedures; and the supernatants were transferred to small screw-cap polyethylene bottles and frozen. At intervals of 3 to 5 days, one urine + standard solution, one urine, and one standard solution were thawed and analyzed. The effect of urine components on recovery was calculated for each acid:

$$\text{recovery from urine} = \frac{[\text{urine + standard solution}] - [\text{urine}]}{[\text{standard solution}]} \times 100\% \quad (2)$$

where [urine + standard solution] = analyzed concentration of resulting ester when urine and acid–salt solution were combined,

[urine] = analyzed concentration of resulting ester of urine and H_2O, and

[standard solution] = analyzed concentration of resulting ester of acid–salt solution and H_2O.

Also, the analyzed ester concentrations of the acid–salt solution were compared to the initial acid or salt concentrations. The same study also was carried out at concentrations of 0.8 and 2.5 µmoles/ml.

Precision. The precision of the method was studied by comparing the results of five replicate analyses in which aliquots of the same urine sample were individually carried through each of the preparatory steps. The mean concentration and standard deviation were calculated for each acid found.

Concentrations in Urine. Seven normal and pathological urine samples were analyzed as described above. The range of concentrations found was calculated for each acid.

RESULTS

Gas Chromatography. One well-separated peak was obtained for the methyl esters of lactate, fumarate, succinate, malate, α-ketoglutarate, and citrate on the DEGS column. However, a "shoulder" was observed on the α-ketoglutarate peak after HCl–methanol esterification. Pyruvate gave two peaks with either method of esterification; the peak areas were

(22) D. Metcalfe and A. A. Schmitz, ANAL. CHEM., 33, 363 (1961).

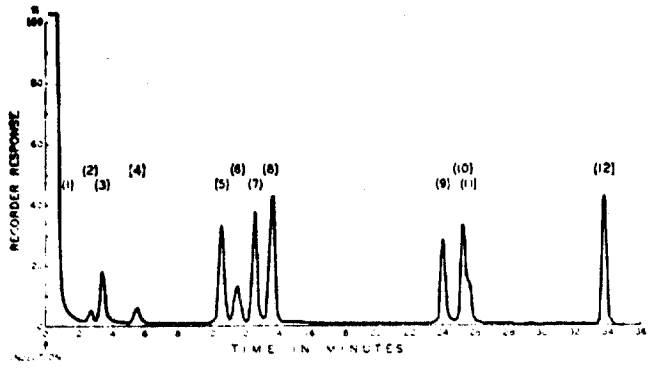

Figure 1. Chromatogram of methyl esters obtained from a solution of pure acids and salts prepared like a urine sample

Separation is on DEGS; sensitivity is 1.09 × 10^{-8}A/full scale; other conditions as in Table I. Identifications are given in the text

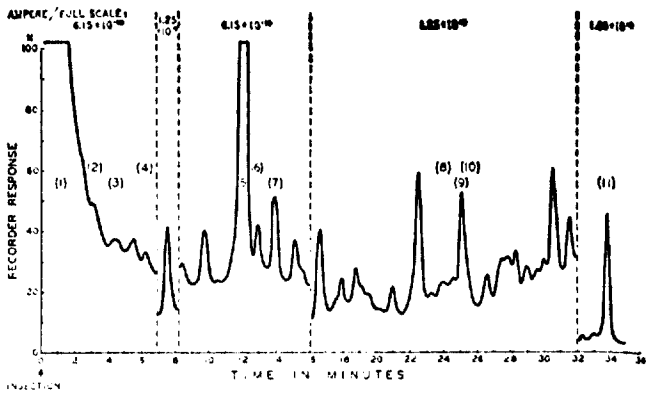

Figure 2. Chromatogram of methyl esters of acids isolated from a normal adult female human urine sample, prepared as described in the text

Separation is on DEGS; sensitivity as indicated; other conditions as in Table I. Tentative identifications are given in the text

combined to estimate the amount of pyruvate. (This combination is strictly valid only if the responses of the two components are identical; otherwise, it is to some extent an approximation. When the compound represented by the second peak, methyl 2,2-dimethoxypropanoate (*11*), is available, its response should be determined and calculations of pyruvate concentration made accordingly.) These esters also were well separated on the XE-60 column, except that lactate and the first pyruvate peak were not resolved. The order of elution was also similar; however, on XE-60 laurate was eluted between malate and α-ketoglutarate. A chromatogram of esters obtained from a mixture of acids and salts, prepared like a urine sample, is illustrated in Figure 1. Peak identifications are as follows: 1) solvent, 2) methyl pyruvate, 3) methyl lactate, 4) methyl 2,2-dimethoxypropanoate (*11*), 5) dimethyl fumarate, 6) esterified benzoic acid, 7) dimethyl succinate, 8) methyl laurate, 9) dimethyl malate, 10) dimethyl α-ketoglutarate (*11*), 11) dimethyl 2,2-dimethoxyglutarate (*11*) and 12) trimethyl citrate. Figure 2 shows a chromatogram from a urine sample. The peaks are tentatively identified as: 1) through 4) as above, 5) esterified benzoic acid, 6) dimethyl succinate, 7) methyl laurate, 8) dimethyl malate, 9) dimethyl α-ketoglutarate (*11*), 10) dimethyl 2,2-dimethoxyglutarate (*11*) and 11) trimethyl citrate. Unidentified peaks are unknown.

Table III. Regression Equations Derived from Response Data

Ester (1 to 25 μmoles/ml)	Pearson's coefficient of correlation	Regression equation ($y = bx + a$)[a] b	a	Standard error of the estimate
Pyruvate	0.999	66.2	6.28	23.7
Lactate	0.997	64.3	49.6	37.8
Fumarate	0.99	130	7.60	146
Succinate	0.99	128	9.78	142
Citrate	0.998	109	68.6	55.9
Citrate (5 to 100 μmoles/ml)	0.99	90.8	37.9	428
Esterified malic acid	0.994	$\log y = 1.23 \log x + 1.55$		0.08

[a] y = peak area in integrator units at attenuation of 6.28 × 10^3. x = concentration of ester (or malic acid) in micromoles per milliliter.

Table IV. Reproducibility of the HCl–Methanol Esterification

Anion	Amount added (μmoles/ml)	Recovery[a] (%)	Range
Pyruvate	8.95	97.3	91.6-104
Lactate	8.63	85.7	84.2- 86.6
Fumarate	8.89	79.8	79.1- 81.3
Succinate	9.10	83.0	82.0- 84.3
Citrate	8.89	81.4	76.7- 85.6

[a] Mean of three determinations.

The reproducibility of the injection was evidenced by the small relative standard deviation of the mean peak area for the nine consecutive injections of methyl lactate solution: 926 units ± 2.37%.

The response of the gas–liquid chromatographic (GLC) system to the methyl esters studied was linear within certain concentrations, but did not extrapolate through the origin. The results are summarized in Table III. The response to trimethyl citrate was also studied at higher concentrations, because higher concentrations are sometimes found in urine. This response was linear in the concentration range of 5 to 100 μmoles/ml, as indicated in Table III.

The use of a concentration/response ratio in calculating unknowns is valid, if the response curve is linear throughout the required range and passes through the origin—*i.e.*, if the regression equation has the form:

$$y = bx \tag{3}$$

in which $b = \dfrac{1}{\text{concentration/response ratio}}$. If the regression is linear but does not pass through the origin—*i.e.*, if the regression equation is:

$$y = bx + a \tag{4}$$

the use of a concentration/response ratio leads to systematic errors in calculating concentrations of the unknown, important especially when the concentrations are low. Consequently, unknowns were calculated from the regression equations, rather than being calculated from response factors.

For the malate and α-ketoglutarate response data, only the log–log malate plot was linear. The results are given at the bottom of Table III.

Since α-ketoglutarate plots were not linear, the acid concentration *vs.* peak area data were used in calculating unknowns. Unknown α-ketoglutarate concentrations were interpolated from the peak area of the unknown and the responses of two known proximate acid concentrations.

Table V. Recovery of Acids and Salts (% of Theoretical)

Anion	A 0.1 μmole/ml[a] of each added		B 0.8 μmole/ml[a] of each added		C 0.8 μmole/ml[a] of each added		D 2.5 μmoles/ml[a] of each added	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Pyruvate	47.6	0–70.2	48.3	45.3–50.9	37.1	32.0–40.5	40.5	30.7–52.2
Lactate	109	88.2–133	65.6	65.3–66.1	79.0	76.2–82.6	79.1	78.1–80.4
Fumarate	81.5	72.0–90.4	76.4	74.9–78.2	81.6	78.1–85.4	90.3	88.9–92.2
Succinate	103	91.7–113	82.5	81.9–83.1	86.0	81.9–89.6	95.9	94.0–98.0
Malate	367	352–388	119	117–121	152	149–158	126	123–128
α-Ketoglutarate	104[b]	104–104[b]	123	119–126	141	126–150	105	95.4–112
Citrate	127	105–159	94.9	91.9–98.4	112	109–115	119	114–124

A, C, D = three aliquots each prepared and chromatographed on different days.
B = three aliquots each prepared on the same day and chromatographed on the same day.
[a] Concentration of the original solution, which is concentrated ten-fold before injection.
[b] Only two determinations.

Table VI. Recovery from Urine (%)[a]

Anion	A No. of determinations	A Mean	A Range	B No. of determinations	B Mean	B Range	C No. of determinations	C Mean	C Range
Pyruvate	2	41.3	35.0–47.4	2	100	94.9–105	3	122	92.9–153
Lactate	3	42.7	34.3–57.1	2	57.9	46.8–69.0	3	93.8	91.7–95.2
Fumarate	2	80.2	73.3–85.0	2	101	94.5–107	3	96.4	93.2–101
Succinate	2	70.1	56.0–77.1	2	98.5	94.0–103	3	98.4	97.1–100
Malate	3	28.9	22.9–31.9	2	77.0	73.6–80.4	3	88.4	86.5–90.4
α-Ketoglutarate	2	141	131–151	2	73.2	63.9–82.4	3	93.8	86.7–107
Citrate	3	64.0	40.4–82.9	2	82	77–86	3	88.6	86.3–92.8

[a] Urine plus acids were concentrated ten times before injection. The following quantities of anion were added to each milliliter of urine:
A = 0.1 μmole
B = 0.8 μmole
C = 2.5 μmoles

Esterification. The HCl–methanol method of esterification yielded ester representing from 79.8 to 97.3% of the starting acid (Table IV). It is difficult to compare these yields, based on amount of ester formed, with those previously reported (*17*), which were based on the amount of acid remaining. The optimal time for BF_3–methanol esterification was found to be between 3 and 4 hours. The HCl–methanol method was preferred, because of the much greater trimethyl citrate yields.

Peak areas of acids and salts esterified with HCl-methanol tended to increase, if the solutions were allowed to stand overnight from the 1st to the 2nd day (except lactate, which decreased slightly), indicating that the esterification reaction was not complete after 4 hours. Variable sensitivity of the instrument was taken into account by noting changes in the response to a methanol solution of pure esters over a 15-day period. The peak areas of pyruvate, lactate, and α-ketoglutarate appeared to be constant from the 2nd through the 15th day. However, after the 2nd day, the peak area of citrate diminished rapidly; and those of fumarate, succinate, and malate also decreased, but more slowly. All urine samples were chromatographed on the day after HCl-methanol esterification.

Recoveries. The mean recoveries of pure acids and salts, following all of the processing steps noted above, varied from 37 to 127%, except for malate, and are shown in Table V. The recoveries of malate, much greater than 100%, indicated some interference, which was particularly evident at low malate concentrations. The low pyruvate values were due to losses during the evaporation step, as evidenced by low pyruvate recoveries obtained when a formic acid solution of acids and salts was evaporated, esterified, and chromatographed. Urine sample results were corrected for recovery of the pure acids and salts.

The effect of urine on recovery is illustrated in Table VI. Substances in the urine particularly interfered with recovery of the oxygenated acids at very low concentrations (0.1 μmole/ml) and with lactate at 0.8 μmole/ml. At higher concentrations there was little interference.

Precision. The relative standard deviation, calculated for each acid for five analyses of the same urine sample, varied from 4.2% at a concentration of 1157 nmoles/ml of citrate to 25.9% at a concentration of 88 nmoles/ml of pyruvate (Table VII). Relative standard deviations over 20% were obtained at very low concentrations (below 100 nmoles/ml). At higher concentrations relative standard deviations decreased.

Concentrations in Urine. Urine concentrations of the acids studied are indicated in Table VIII.

DISCUSSION

Gas Chromatography. As recommended by the manufacturer for maximum sensitivity, extra helium was added to the carrier effluent, making the flow at the burner tip 120 ml/min (see Table I).

An isothermal column temperature was not satisfactory for the separation of these esters, because of the wide range of boiling points. Nor was a single linear temperature program applicable, because the peaks were eluted in pairs; and either the time between pairs, and consequently the analysis time, was too long; or the peaks within a pair were not resolved. A multilinear temperature program (Table I) was found to be satisfactory. This program precluded use of the Kovats Re-

Table VII. Reproducibility of Five Replicate Analyses of TCA Cycle and Related Acids in Human Urine[a]

Anion	Nanomoles/ml[b] #1	#2	#3	#4	#5	Mean	S.D.	S.E.	Relative S.D. (%)
Pyruvate	110	80.0	78.0	113	59.5	88.1	22.8	10.2	25.9
Lactate	44	45.3	53.5	41	65.5	49.9	9.9	4.4	20
Succinate	11	7.0	7.5	9.5	13.1	9.6	2.5	1.0	25
α-Ketoglutarate	237	171	166	170	166	182	31	14	17
Citrate	1077	1181	1202	1155	1172	1157	48	22	4.2

[a] The urine specimen was a single voiding, collected in a chilled container from a hospitalized child. Five aliquots were taken and processed separately.
[b] Calculated concentrations in the urine; concentrations injected were twenty times these.

Table VIII. Range of Concentrations of TCA Cycle and Related Acids in Seven Samples of Human Urine

Anion	Nanomoles/ml
Pyruvate	59.0–189
Lactate	53.5[a]–81.0
Fumarate	0–30.3
Succinate	23.0–85.6
Malate	13.5–112
α-Ketoglutarate	74.5–499
Citrate	65–3250[b]

[a] Four urines contained less than 53.5 nmoles/ml, which could not be measured.
[b] One urine contained more than 5050 nmoles/ml, which could not be measured.

tention Index for identification, proposed for isothermal systems (*23*) and later extended for use with linear temperature programming (*24*). The relative retention was, therefore, used as the basis for identification, with methyl laurate as an internal standard, because it is stable and was eluted approximately midway through the program.

Several compounds were esterified and chromatographed to determine whether a peak which interfered with the analysis would be obtained. No interfering peaks were observed on either DEGS or XE-60 for the following: creatine, creatinine, tartaric acid (tested on DEGS only), perchloric acid, alanine, arginine, aspartic acid, cystine, glycine, histidine·HCl·H_2O, hydroxyproline, leucine, lysine·HCl, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. Glutamic acid, glutamine, and methionine gave very broad peaks on XE-60. However, these appeared after the citrate peak and did not interfere with the analysis. As expected, peaks were observed for several carboxylic acids on DEGS, including glycolic, oxalic, malonic, and adipic. A peak also was noted for trichloroacetic acid, a common deproteinizing agent, on both columns. Methyl lactate and trichloroacetic acid were indistinguishable on DEGS; therefore, $HClO_4$, rather than CCl_3COOH, was used for deproteinization. Also, commercially obtained methyl esters of the saturated, straight-chained fatty acids C_6 and C_8 through C_{19} gave well-separated peaks on both columns. However, some overlapping with acids being studied was observed. Because these fatty acid methyl esters represented only a few of the many unknown acids which may appear in urine, it has been necessary to confirm identifications on at least one other column. The XE-60 column has been used for this purpose. An alternative confirmation procedure would be to compare retention data of two derivatives (*21*). The trimethylsilyl ester, originally proposed by Horii (*10*), was applied with some success (*12*, *21*). We have not yet investigated this possibility.

As previously noted, pyruvate gave rise to two peaks with either esterification procedure; the first of which has been identified as methyl pyruvate by retention data. This phenomenon, following HCl–methanol esterification, was previously reported (*8*, *11*); and the first peak also has been identified as methyl pyruvate using other analytic techniques (*11*). These two peaks were not due to instrumental conditions, as suggested by Estes and Bachmann (*16*), because the commercial ester in methanol solution gave only the first peak. Thus, it also seems the two peaks are not due to a spontaneous dissociation and equilibrium between two forms of the ester in solution. Extracting the reaction solution with chloroform increased the second peak, while the first peak disappeared, supporting the possibility of interconversion. As previously suggested (*16*), this interconversion seems to be the reason for the single pyruvate peak, eluted after lactate, observed by Alcock (*14*). The second peak has been identified as methyl 2,2-dimethoxypyruvate (*11*). A single pyruvate peak was observed by Gee, using HCl–methanol–thionyl chloride esterification (*9*), and by others, using diazomethane esterification (*16*, *20*). However, Simmonds *et al.* (*11*) reported two peaks for pyruvate with diazomethane; the first, methyl pyruvate, was rapidly converted to the second, methyl 2-methylglycidate. Presumably, the single "pyruvate" peak finally obtained by this esterification is not methyl pyruvate. A single peak was obtained by Horning *et al.* (*12*) for the trimethylsilyl ester, when a derivative of the keto group also was formed.

Esterification of α-ketoglutaric acid yielded one peak with BF_3–methanol and two peaks (unresolved on DEGS) with HCl–methanol. Two peaks for α-ketoglutarate with HCl–methanol were previously observed (*11*). The first was identified as the dimethyl ester; the second as dimethyl 2,2-dimethoxyglutarate, by Simmonds *et al.* (*11*). Multiple peaks for α-ketoglutarate, esterified with diazomethane, also were reported (*11*, *15*); the first was identified as the dimethyl ester and the second as 2-(carbomethoxyethyl)-glycidate (*11*). When a derivative of the keto group also was formed, a single peak was obtained for the trimethylsilyl ester (*12*).

Oxaloacetic acid, at about 25 μmoles/ml, when esterified with HCl–methanol yielded only two very small peaks, tentatively identified as pyruvate, which account for less than 5% of the acid. This may complicate interpretation of pyruvate analyses but seems minimal.

Trans-aconitate, after HCl–methanol esterification, yielded one peak eluted just after α-ketoglutarate on DEGS, as previously reported (*18*). *Cis*-aconitate, similarly treated, yielded three peaks: the first in order of elution had the same relative

(23) E. Kovats, *Helv. Chim. Acta*, **41**, 1915 (1968).
(24) H. Van den Dool and P. D. Kratz, *J. Chromatogr.*, **11**, 463 (1963).

retention as the trans peak, and the third was eluted just before citrate. The peaks, listed according to size, were: 2 > 1 > 3.

When either isocitric acid lactone or trisodium isocitrate was esterified with HCl-methanol in the usual way, a very small succinate peak (tentative identification), accounting for less than 3% of the isocitrate, and a small peak, eluted just after citrate, which increased after refrigeration for 10 days to account for about 20% of the starting material, were observed. The 20% estimate was based on citrate response. The error in succinate analysis, due to the succinate produced, would probably be minimal.

Barium oxalosuccinate, when esterified with BF_3–methanol, gave three main peaks, the GLC of which has not been previously reported: the largest was tentatively identified on DEGS as α-ketoglutarate; one of the other two, which were much smaller, was tentatively identified as succinate. The remaining small peak was eluted just after the second pyruvate peak and was similar in retention to oxalate. It seems, therefore, that high concentrations of oxalosuccinate would distort α-ketoglutarate and, possibly, succinate analyses.

Due to the formation of multiple peaks or to the failure to produce a characteristic peak, oxaloacetate, *cis*-aconitate, isocitrate, and oxalosuccinate were not included in the present quantification.

Isolation of Acids from Urine. In order to denature enzymes, which could modify the original TCA cycle acid content, urine was collected over ice and kept cold until deproteinized. Although the Dowex 2 exchange capacity and rate are considered to be independent of pH (*4*), we noted that very low recoveries were obtained when neutralization was omitted following acid deproteinization. This may have been due to high perchlorate ion concentration in the unneutralized sample. Neutralization with KOH precipitated the perchlorate. Samples were adjusted to pH 5 before ion exchange, to ensure reproducible recoveries.

Because TCA cycle and related acids are excreted in relatively low concentration in the urine, it was necessary to concentrate the acids for GLC analysis. After evaporation of the urine, the large amount of gum-like residue could not be dissolved in small amounts of solvent. Ether extraction alone has been reported to be inadequate. Because of the variety of components in urine, further purification was necessary (*13*). Organic acids have been extracted more completely by ion exchange than by ether extraction (*4*). In particular, ether extraction of citrate was incomplete (*21*); and ion exchange was recommended for such polyfunctional acids (*19*).

A method of isolation proposed by Alcock (*14*), based on exhaustive ether extraction, which also required adsorption of the acids on acid alumina and elution with BF_3–methanol, was rejected. Recoveries, which included prior deproteinization and neutralization, were inconsistent in our hands; and the substantial volume of BF_3–methanol required to elute the acids from the alumina made further concentration of the effluent necessary.

An ion exchange procedure appeared to be the most satisfactory for our purpose. Dowex resin has been used to isolate acids from biological fluids for gas chromatography (*20*). Formation of lactic acid from sugar breakdown, observed on a Dowex 2 resin, was not noted with Dowex 2 in the formate form (*4*). However, the ion exchange procedure did introduce an unknown peak in the gas chromatogram between dimethyl fumarate and dimethyl succinate. A similar unknown peak was reported after ion exchange and subsequent gas chromatography (*15*). When benzoic acid was esterified and chromatographed on DEGS, the peak obtained had the same relative retention as the unknown peak, which, consequently, was tentatively identified as the esterification product of benzoic acid. This benzoic acid may be a product of the breakdown of the Dowex 2 resin, which has a polystyrene lattice, in the presence of the formic acid. Isolations with several other, smaller columns were attempted; but recoveries were low. The resin was purchased in the formate form, because attempted conversions from the chloride form were unsatisfactory. A 1.5*N* ammonium carbonate solution had been used as an ion exchange eluent (*25*). The effluent was subsequently evaporated to dryness in a rotary evaporator at 60 °C, thereby decomposing and removing the $(NH_4)_2CO_3$. When a solution of the acids used in this study was prepared in 1.5*N* $(NH_4)_2CO_3$, evaporation, esterification of the residue, and gas chromatography yielded only a small citrate peak, indicating a loss of most of the acids.

Esterification. The low volatility and thermal decomposition of some of the acids being studied necessitated the formation of a derivative prior to gas chromatography (*18*). The increased volatility and stability, ease of formation, and commercial availability of methyl esters was advantageous. Solvent evaporation after esterification was avoided to reduce losses of the more volatile esters (*13*, *20*). Repeated extractions with chloroform were incomplete; and the pyruvate peaks were disturbed, as described above. Consequently, chloroform extraction of the esterification reaction mixture was rejected; and the reaction mixture itself was chromatographed.

CONCLUSION

Although gas chromatography itself is rapid, it effects a simultaneous analysis of several compounds in a very small aliquot, and may be particularly useful in working with radioactively labeled compounds, GLC analyses of biological material is difficult. The difficulty stems from the complexity of biological samples on the one hand and their often extremely low concentration on the other. With pure esters the gas chromatographic system gives reproducible results, and correlation coefficients calculated for response data are close to unity.

The preparatory steps necessitate large sample volumes (10–20 ml) and result in some low and/or variable recoveries. Incomplete and/or nonreproducible ester formation does not seem responsible for these low and/or variable recoveries. Apparently the losses are incurred during the ion exchange and subsequent evaporation procedures. The method reported in this paper is applicable to urine as described. It will be more accurate, precise, and useful, when the preparatory steps are improved or can be deleted.

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(25) F. E. Resnick, L. A. Lee, and W. A. Powell, ANAL. CHEM., 27, 928 (1955).

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Enzymatic determination of L-malic acid in the blood

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Malic acid in the blood has not, until now, been measured by anyone except Hummel /1/ with the aid of a fluorimetric technique involving condensation in homo-ombelliferone (7-hydroxy-5-methyl-coumarine) of malic acid with orcinol in sulphuric medium. But this method lacks specificity, and the results obtained in its use can be fraught with errors due to interference of substances where the structure is very different from that of the malic acid. Moreover, it also determines the L-malic isomer a constituent of the tricarboxylic cycle, and the D-malic isomer whose place in metabolism has been specified in recent works /2,3/.

We will describe here an enzymatic technique which permits the determination of just the L-malic isomer in the blood; the results thus obtained have been compared with those of measurements made in parallel by Hummel's technique /1/.

The principle which we have applied to the enzymatic determination of L-malic acid in the blood is identical to that used by Hohorst et coll. /4/, for the measurement of L-malic acid in the liver of the rat, but different modalities had to be developed by reason of the very low concentration of L-malic acid in the blood, and particularly in the blood plasma.

The unfavorable equilibrium /5/ of the reaction:

L-malic acid +NAD forms and is formed by oxalo acetic acid + NADH + H^+ catalyzed by L-malate : NAD oxydoreductase (1.1.1.37(**); malicodehydrogenase), is modified by addition of a substance such as hydrazine sulphate which reacts with oxalo acetic acid. Under these conditions, and with a pH of 9.6, the reaction is totally deviated from left to right, so that there is equality between the number of molecules of L-malic acid initially present and

that of $NADH_2$ formed. The latter is determined thanks to the variation in optical density at 340 m mu according to Warburg's optical test.

The adjunction of hydrazine sulphate or some other reagent of the carbonyl group is, however, responsible for a certain number of technical difficulties which we shall analyze below. Similar difficulties are encountered in the enzymatic determinations of lactic acid /6/ and beta-hydroxy butyric acid /7/ in the blood, but they are particularly sensitive during the determination of L-malic acid, because the concentration in the blood is much smaller than that of lactic or beta-hydroxy-butyric acid.

Technique

Description of the technique

The determination is made by successively introducing, into 2 tanks of quartz, 1 cm thick, 2.5 ml of the sample to be analyzed (containing 2 x 10^{-3} to 2 x 10^{-2} mu moles of L-malic acid); 0.45 ml of a glyco-glue/hydrazine sulphate/soda buffer (final concentrations, respectively : 1 M/0.1 M/0.274 M) (pH : 9.6), then, 15 minutes later, 50 mu l of a 6 x 10^{-2} M solution of NAD^+ (Boehringer).

We then add, to one of the two tanks, 20 mu l of a preparation of malico dehydrogenase (pork heart extract) in solution (0.5 mg/ml) in glycerol at 30%, and having a specific activity /8/ of 7500 units/mg (Boehringer).

We then compare, with the aid of a Beckman DU spectrophotometer, the absorption at 340 m mu of this test tank with that of the control tank (containing no enzyme(, the readings being made very 5 minutes until a level, stable for 20 minutes, is obtained.

The concentration of L-malic acid in the sample is calculated from the difference in optical density between the two tanks at the moment this level is obtained, with consideration of a coefficient of extinction for the $NADH_2$ of 6.22 cm^2/mu hole at 340 m mu /9/.

The application of this technique to 10 samples of 2.5 ml of control solutions containing 4 x 10^{-3} to 8 x 10^{-3} mu moles/ml of L-malic acid (Eastman

Kodak) gave us a recovery of 97 ± 3%.

Application of the method to blood

(1) Choice of anticoagulant and sampletaking

The blood taken by venous puncture is collected on sodium fluoride (1.5 mg/ml of blood) and heparin (0.25 mg/ml of blood). We verified that such concentrations of fluoride and heparin do not inhibit the enzymatic dehydrogenation of L-malic acid.

For determinations in plasma and the red corpuscles, the blood is centrifuged at +4°C. If this centrifugation cannot be done immediately, the blood can be kept for 2 hours at +4°C without alteration of the concentration of L-malic acid in the plasma and the red corpuscles.

(2) Deproteinization

Deproteinization is carried out by addition of an equal volume of perchloric acid (30% p/v) for the whole blood and 6% for plasma. In the case of the red blood cells, the hemolysis is carried out by addition of 4 volumes of distilled water for 3 volumes of red cells, then freezing at -10°C, and thawing; we then add a volume of 6% perchloric acid equal to that of the red blood cell hemolysate.

After centrifugation, an aliquot part of the supranatant liquid is alkalinized to a pH of 9.6 by addition of 2 N KOH, with notation of the volume of KOH used. After 30 minutes at +4°C, the precipitate of potassium perchlorate is eliminated by centrifugation and the determination of L-malic acid is made on 2.5 ml of the supranatant. The latter can be kept for several days at +4°C without alteration of the percentage of L-malic acid.

(3) Reproducibility of the results.

Making ten determinations of L-malic acid on a given plasma, we found, for an arithmetic mean of 0.255 mu mole/100 ml, a value for (2 sigma)/square root of N, of ± 0.0096, (sigma being the estimated typical spread, and N the number of determinations). The extreme values obtained during these

determinations are 0.242 and 0.287 mu mole/100 ml.

(4) Recovery of the L-malic acid added to the plasma

On adding 4×10^{-3} to 15×10^{-3} mu moles of L-malic acid per ml of plasma to plasmas whereof the L-malic acid concentration was previously determined, the results obtained during the enzymatic measurement of this acid indicate a recovery of 97.8 $\pm$ 3.9% (arithmetic mean $\pm$ (2 sigma/square root of N) (Table I).

(5) Specificity

D-malic acid is not dehydrogenated by the enzymatic preparation used, and its presence, in a quantity equal to that of the L isomer does not interfere with the determination of the latter. Thus the application of the technique described, to a control solution containing 10^{-2} mu mole/ml of DL-malic acid yields results corresponding to the L-malic isomer content of this solution (5×10^{-3} mu mole/ml.

Table I. Recovery of L-malic acid added to the blood plasma

Column I. Determination of L-malic acid in plasma before adjunction (in mu moles/100 ml)

II. Quantity of L-malic acid added to the plasma (in mu moles/100 ml)

III. Determination of L-malic in plasma complemented by L-malic acid (in mu moles/100 ml)

IV. Percentage of recovery of L-malic acid)

I	II	III	IV.
0.309	0.400	0.784	110.6 p. 100
0.317	1.400	0.717	100.0 p. 100
0.329	0.400	0.690	94.7 p. 100
1.032	0.400	1.380	96.2 p. 100
0.329	0.800	1.080	95.7 p. 100
0.331	0.800	1.089	96.1 p. 100
0.332	0.800	1.042	92.0 p. 100
0.329	1.500	1.775	97.2 p. 100

The presence in the blood of lactic acids and beta-hydroxy butyric acids does not interfere with the determination of L-malic acid under the conditions described. As a matter of fact, we have observed no alteration of the optical density at 340 m mu in the course of the addition of malico dehydrogenase, on replacing the blood defectate by solutions of lithium lactate or sodium beta-hydroxy-butyrate whose concentrations correspond to the maximum figures described for these 2 acids in normal blood.

Discussion of the technique

In order that the alteration in optical density, after addition of the enzyme, will be of sufficient amplitude to permit precise readings, we were led, in view of the low concentration of malic acid in the plasma, to use a volume of defecate (2.5 ml) much larger than that usually called upon for enzymatic analyses based on the Warburg optical test.

The resulting drawback is that the concentration of interfering substances present in the plasma is likewise increased.

Thus, after addition of the glyco glue/hydrazine sulphate/soda buffer, there is a gradual drop in the optical density, followed, after about 15 minutes, by a stabilization of the latter. This drop. apparently due to an interaction between the hydrazin and certain constituents of the blood de-

fecate, led us to wait 15 minutes between the addition of the buffer and that of the NAD^+.

The alterations in optical density consecutive to the addition of NAD^+ consist in an immediate rise, followed by a more discrete but gradual elevation, varying in amount according to the defecates. They are apparently due to the non-enzymatic formation of a complex of addition between the NAD^+ and the reagent from the carbonyl group /6, 10/.

In looking for the factors enabling us to reduce to a minimum these alterations in optical density supervening before the addition of malico dehydrogenase, we were led to substantially reduce the hydrazine sulphate concentration relative to that recommended by Hohorst et coll. /4/. Under these conditions, the variations in optical density remain minimal; we nevertheless eliminate their influence by using, as control, a tank containing all the reagents except the enzyme. It should be noted that these variations in optical density cannot be eliminated either by substitution of the As_2O_3/NaOH buffer /6/ by the glyco glue/soda buffer, nor by addition of ethylene-diamine-tetra acetic acid in the final concentration of 0.75 mu mole/ml.

The dehydrogenation of the malic acid under the conditions described, is relatively slow, but is not accompanied by an alteration in the enzyme, as proved by the complete dehydrogenation of a supplementary quantity of L-malic acid added at the end of the reaction. We checked whether it was possible to accelerate this reaction by increasing the quantities of enzyme and of NAD^+. It was thus found that the speed of the reaction was not substantially altered by a sharp increase in the quantity of enzyme (up to 375 units). The increase in the concentration of NAD^+, however, does accelerate the reaction, but also accentuates the secondary reactions due to interfering substances, which led us to avoid exceeding the quantity of NAD^+ indicated in the description of the technique (3 mu moles).

As for the determination of the end of the reaction, we estimate, as indicated above, the dehydrogenation of the L-malic acid is terminated when the difference in optical density between the 2 tanks remains stable for 20 minutes. In certain cases we observe a later reduction in the difference between the 2 tanks, the optical density of the test tank tending to meet that of the control tank. Hohorst /11/ during the determination of lactic acid, describes an analogous phenomenon which he attributes to a partial reoxidation of the $NADH_2$, ending in the formation of an unidentified compound, having maximum absorption at 400 m mu. This reoxidation can be prevented either by increasing the concentration of enzyme, or using a measuring device sheltered from the air. Such technical modalities are generally useless, however in the determination of L-malic acid in the blood, because it is only in exceptional cases that we did not observe a parallelism between the optical densities of the test tank and of the control tank during a time long enough to permit fixing the moment of the end of the reaction.

Comparison with the fluorimetric method

We determined, in parallel with the enzymatic measurements, the malic acid in the blood, with the aid of a Photovolt fluorimeter with Hummel's technique /1/, modified only in respect of the volume of the sample test (1 ml in lieu of 0.2 ml).

Results

The proportion of L-malic acid in whole human blood, found in 13 healthy, fasting subjects, is 1.173 $\pm$ 0.270 mu moles/100 ml (arithmetic mean $\pm$ (2 sigma)/ square root of N) by the enzymatic method, while the result of the analyses made in parallel with Hummel's technique /1/ is 1.93 $\pm$ 0.20 mu moles/ 100 ml.

It appears, therefore, that the values obtained with the aid of the enzymatic method described are much smaller than those of the fluorimetric technique, which speaks for the better specificity of the former.

The proportion of plasmatic malic acid determined by the enzymatic method

in 40 healthy, fasting subjects if 0.413 ± 0.035 mu moles/100 ml. We did not observe any significant differences according to sex or age.

In the case of plasma, as in the case of whole blood, the results obtained in parallel by the fluorimetric method are much higher (0.585 ± 0.048 mu moles/100 ml.

The ascertainment of a much lower proportion of L-malic acid in plasma than in whole blood led us to practice the enzymatic determination of this acid in the red blood corpuscles. We found here a concentration of 2.40 plus or minus 0.55 mu moles/100 ml (mean of 8 determinations).

The mean proportions found, respectively, in the whole blood, plasma and red cells thus appear to be concordant, with consideration of a normal figure of about 40% for the red cell.

Discussion

The normal concentration of L-malic acid in human whole blood (mean of 1.173 mu moles/100 ml) is much smaller than that of pyruvic acid (8.75 mu moles/100 ml according to Strohmeyer et coll. /12/) and citric acid (9.6 mu moles/100 ml according to Woolcott and Boyer /13/). But it is on the same order of size as that of alphaketoglutaric acid (0.96 mu moles/100 ml per Strohmeyer et coll. /12/). It is also close to the normal proportion found by Grundig /14/ for oxalo acetic acid (1.36 mu moles/100 ml); for the latter acid, however, the results found by Keller and Denz (2.86 mu moles/100 ml) /15/, are substantially higher.

The distribution of malic acid between plasma and red blood cells appears remarkable inasmuch as human red cells are much richer than plasma in this acid, while they are poorer in citric acid /16/, and since the globular and plasmatic concentrations are substantially identical in the case of pyruvic and alpha-keto glutaric acids, as witnessed by the comparison of the results relative to whole blood on theone hand, and plasma or serum on the other /12, 17, 18/.

The relative richness of human red blood cells in L-malic acid raises the problem of the origin of this acid.

Mature red blood cells of mammals have no functional tricarboxylic cycle /19, 20/. The maturation of the reticulocyte to a normocyte is accompanied, as a matter of fact, by the almost total disappearance of the activity of the citrate hydro-lyase (4.2.1.3; aconitase) and the succinate : (acceptor) oxydoreductase (1.3.99.1; succino dehydrogenase) /21/. In the absence of an active citric cycle in the normocytes, the metabolism of the glucose by way of glycolysis leads here to an accumulation of pyruvate /22/ and lactate /23, 24/.

Malate apparently forms from these terminal products of glycolysis. Since Rubinstein and Denstedt /24/ were unable to demonstrate L-malate : NADP oxydoreductase (decarboxylant) (1.1.1.40) in the red cells, it is probable that the malate is formed therein thanks to a GTP; oxalo acetate carboxy-lyase (4.1.1.32) by carboxylation of phospho-enol-pyruvate into oxalo acetate, followed by reduction of the oxalo acetate into malate. The latter is done thanks to the L-malate : NAD oxidoreductase (1.1.1.37 ; malico dehydrogenase), the persistance of which in the mature globules of the mammals was verified by many authors /21/.

The rise in the intra-erythrocytary concentration of the malate, which we have observed, in the course of preliminary experiments, by incubating human red blood cells with glucose, is in favor of such an endo-globular synthesis of the malate from intermediaries formed in the course of the glycolysis. This synthesis may represent the inverse of the transformation of the malate into lactate, a transformation which was proven in the red blood cells of mammals by Spicer & Clark /25/.

The role of endo-globular synthesis of malate appears to be two-fold. On the one hand, the carboxylation of the phospho-enol-pyruvate into oxalo acetate requiring GTP (or ITP), which is itself formed from ATP thanks to the ATP : nucleoside/diphosphate phospho transferase (2.7.4.6), would consume the ATP, whereof the adult red blood cell produces an excess /20/. On the other

hand, the reduction of the oxalo acetate into malate, accompanied by a reoxidation of the $NADH_2$ produces, in the course of the dehydrogenation of the 3-phospho-glyceraldehyde, would permit in parallel to the reduction of the pyruvate into lactate, avoiding an accumulation of $NADH_2$.

We wish to thank the National Institute of Hygiene (Director: Prof. L. Bugnard), for their support of this work.

(Resumes in French, English and German - see pages 810 - 811)

Bibliography

(See page 811)

Footnotes:

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Abbreviations used:

NAD^{plus} or NDA : Nicotinamide-adenine-dinucleotide (oxidized)

$NADH^{plus}{}_{H^{plus}}$ of $NADH_2$: Nicotinamide-adenine-dinucleotide reduced.

NADP : Nicotinamide-adenine-cinucleotide-phosphate.

ATP : Adenosine-tri phosphoric acid

GTP : Guanosine-tri phosphoric acid

ITP : Inosine-tri phosphoric acid

(**) The figures following the names of the enzymes correspond to the nomenclature established in 1961 by the Commission on enzymes of the Union Internationale de Biochimie.

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Die Hemmungen durch die Cl^-, $So_4^=$, $PO_4^=$ Ionen und durch die nicht hydrolisierbaren Steroidsulfate wurden untersucht.

Die Struktur der Steroide bestimmt die Affinität der Enzyme. Die Steroidsulfate mit einer 3β, 5αoder, 3β, Δ_5 Struktur werden schneller hydrolisiert, ebenso die œstrogensulfate und die Corticosteroide.

Die Affinität der Steroidsulfate mit axialer Struktur ist für die 3β, 5β Arten schwach, völlig negativ für die 3α, 5α Arten.

Die Spezifität der Steroidsulfatase von *Helix pomatia* ist grösser als die des entsprechenden Enzyms von *Patella*.

Es zeigt sich, dass das gleiche Enzym die 3β-Steroidsulfate und das Cortisonsulfat hydrolisiert.

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DOSAGE ENZYMATIQUE DE L'ACIDE L-MALIQUE SANGUIN.

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(Mémoire reçu le 15 avril 1963).

L'acide malique sanguin n'avait été dosé jusqu'ici chez l'homme que par Hummel [1] à l'aide d'une technique fluorimétrique, comportant la condensation en homo-ombelliférone (7-hydroxy-5-méthyl-coumarine) de l'acide malique avec l'orcinol en milieu sulfurique. Cette méthode manque cependant de spécificité et les résultats obtenus lors de son utilisation peuvent être entachés d'erreurs dues à l'interférence de substances dont la structure est très différente de celle de l'acide malique. De plus, elle dose à la fois l'isomère L-malique, constituant du cycle tricarboxylique, et l'isomère D-malique, dont certains travaux récents [2, 3] ont précisé la place dans le métabolisme.

Nous décrivons ici une technique enzymatique permettant la détermination dans le sang du seul isomère L-malique ; les résultats ainsi obtenus ont été comparés à ceux de dosages faits parallèlement par la technique de Hummel [1].

Le principe que nous avons appliqué à la détermination enzymatique de l'acide L-malique sanguin est identique à celui utilisé par Hohorst et coll. [4] pour le dosage de l'acide L-malique dans le foie de rat, mais des modalités différentes ont du être mises au point en raison de la très faible concentration de l'acide L-malique dans le sang, et notamment le plasma sanguin.

L'équilibre défavorable [5] de la réaction :

Acide L-malique + $NAD^+ \rightleftharpoons$ Acide oxaloacétique + NADH + H^+ catalysée par la L-malate : NAD oxydoréductase (1.1.1.37 (**) ; malico-

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Abréviations utilisées :
NAD^+ ou NAD : Nicotinamide-adénine-dinucléotide (oxydé).
NADH + H^+ ou $NADH_2$: Nicotinamide-adénine-dinucléotide réduit.
NADP : Nicotinamide-adénine-dinucléotide-phosphate.
ATP : Acide adénosine-triphosphorique.
GTP : Acide guanosine-triphosphorique.
ITP : Acide inosine-triphosphorique.

(**) Les chiffres indiqués après les noms d'enzymes correspondent à la nomenclature établie en 1961 par la Commission sur les enzymes de l'Union internationale de Biochimie.

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déshydrogénase) est modifié par addition d'une substance, telle que le sulfate d'hydrazine, qui réagit avec l'acide oxaloacétique. Dans ces conditions et à pH 9,6, la réaction est totalement déviée de gauche à droite, si bien qu'il y a égalité entre le nombre de molécules d'acide L-malique initialement présent et celui de $NADH_2$ formé. Ce dernier est déterminé grâce à la variation de densité optique à 340 mμ, selon le test optique de Warburg.

L'adjonction de sulfate d'hydrazine ou d'un autre réactif du groupement carbonyle est cependant responsable d'un certain nombre de difficultés techniques, que nous analyserons plus loin. Des difficultés analogues se rencontrent lors des dosages enzymatiques des acides lactique [6] et β-hydroxybutyrique [7] dans le sang, mais elles sont particulièrement sensibles lors de la détermination de l'acide L-malique, dont la concentration sanguine est beaucoup plus faible que celle des acides lactique ou β-hydroxybutyrique.

TECHNIQUE.

Description de la technique.

Le dosage est effectué en introduisant successivement dans 2 cuves en quartz de 1 cm d'épaisseur 2,5 ml de l'échantillon à analyser (contenant de 2×10^{-3} à 2×10^{-2} μMoles d'acide L-malique) ; 0,45 ml d'un tampon glycocolle/sulfate d'hydrazine/soude (concentrations finales respectives : 1 M/0,1 M/0,274 M) (pH : 9,6), puis, quinze minutes plus tard, 50 μl d'une solution 6×10^{-2} M de NAD^+ (Boehringer).

On ajoute alors dans l'une des 2 cuves 20 μl d'une préparation de malicodéshydrogénase (extraite de cœur de porc) en solution (0,5 mg/ml) dans du glycérol à 30 p. 100 et ayant une activité spécifique [8] de 7500 unités/mg (Boehringer).

L'on compare alors, à l'aide d'un spectrophotomètre Beckman DU, l'absorption à 340 mμ de cette cuve-essai à celle de la cuve-témoin (ne contenant pas d'enzyme), les lectures étant faites toutes les 5 minutes, jusqu'à obtention d'un palier stable pendant 20 minutes.

La concentration de l'acide L-malique dans l'échantillon est calculée d'après la différence de densité optique entre les 2 cuves au moment de l'obtention de ce palier, en tenant compte d'un coefficient d'extinction du $NADH_2$ de 6,22 $cm^2/\mu Mole$ à 340 mμ [9].

L'application de cette technique à 10 échantillons de 2,5 ml de solutions témoins contenant de 4×10^{-3} à 8×10^{-3} μMoles/ml d'acide L-malique (Eastman Kodak) nous a donné une récupération de 97 ± 3 p. 100.

Application de la méthode au sang.

(1) Choix de l'anticoagulant et prélèvement.

Le sang, prélevé par ponction veineuse, est recueilli sur fluorure de sodium (1,5 mg/ml de sang) et héparine (0,25 mg/ml de sang). Nous

avons vérifié que de telles concentrations de fluorure et d'héparine n'inhibent pas la déshydrogénation enzymatique de l'acide L-malique.

Pour les dosages dans le plasma et les hématies, le sang est centrifugé à + 4°C. Si cette centrifugation ne peut être réalisée immédiatement, le sang peut être conservé pendant 2 heures à + 4°C. sans modification de la concentration en acide L-malique du plasma et des hématies.

(2) Déprotéinisation.

La déprotéinisation est réalisée par addition d'un volume égal d'acide perchlorique à 30 p. 100 (p./v.) pour le sang total, à 6 p. 100 pour le plasma. Dans le cas des hématies, l'hémolyse est effectuée par addition de 4 vol. d'eau distillée pour 3 vol. d'hématies, puis congélation à —10°C. et décongélation ; on ajoute alors un volume d'acide perchlorique à 6 p. 100 égal à celui de l'hémolysat d'hématies.

Après centrifugation, une partie aliquote du liquide surnageant est alcalinisée jusqu'à pH 9,6 par addition de KOH 2 N en notant le volume de KOH utilisé. Après 30 min. à + 4°C., le précipité de perchlorate de potassium est éliminé par centrifugation et le dosage de l'acide L-malique est effectué sur 2,5 ml du surnageant. Ce dernier peut être conservé plusieurs jours à + 4°C. sans que sa teneur en acide L-malique se modifie.

(3) Reproductibilité des résultats.

En effectuant dix dosages d'acide L-malique sur un même plasma, nous avons trouvé, pour une moyenne arithmétique de 0,255 μMol./100 ml, une valeur de $\frac{2\sigma}{\sqrt{N}}$ de ± 0,0096 (σ étant l'écart-type estimé et N le nombre de dosages). Les valeurs extrêmes obtenues lors de ces dosages sont de 0,242 et 0,287 μMol./100 ml.

(4) Récupération de l'acide L-malique ajouté au plasma.

En ajoutant de 4×10^{-3} à 15×10^{-3} μMoles d'acide L-malique par ml de plasma à des plasmas dont la concentration en acide L-malique avait été précédemment déterminée, les résultats obtenus lors du dosage enzymatique de cet acide indiquent une récupération de 97,8 ± 3,9 p. 100 (moyenne arithmétique $\pm \frac{2\sigma}{\sqrt{N}}$) (tableau I).

(5) Spécificité.

L'acide D-malique n'est pas déshydrogéné par la préparation enzymatique utilisée et sa présence, en quantité égale à celle de l'isomère L, n'interfère pas avec le dosage de ce dernier. C'est ainsi que l'application de la technique décrite à une solution témoin contenant 10^{-2} μMol./ml d'acide DL-malique donne des résultats correspondant à la teneur de cette solution en isomère L-malique (5×10^{-3} μMol./ml).

TABLEAU I.

Récupération de l'acide L-malique ajouté au plasma sanguin.

Dosage de l'acide L-malique dans le plasma avant adjonction (en µMoles/100 ml)	Quantité d'acide L-malique ajoutée au plasma (en µMoles/100 ml)	Dosage de l'acide L-malique dans le plasma additionné d'acide L-malique (en µMoles/100 ml)	Pourcentage de récupération de l'acide L-malique
0,309	0,400	0,784	110,6 p. 100
0,317	0,400	0,717	100,0 p. 100
0,329	0,400	0,690	94,7 p. 100
1,032	0,400	1,380	96,2 p. 100
0,329	0,800	1,080	95,7 p. 100
0,331	0,800	1,089	96,1 p. 100
0,332	0,800	1,042	92,0 p. 100
0,329	1,500	1,775	97,2 p. 100

La présence dans le sang des acides lactique et β-hydroxybutyrique n'interfère pas avec le dosage de l'acide L-malique dans les conditions décrites. Nous n'avons, en effet, observé aucune modification de la densité optique à 340 mµ lors de l'addition de malicodéshydrogénase en remplaçant le défécat sanguin par des solutions de lactate de lithium ou de β-hydroxy-butyrate de sodium dont les concentrations correspondaient aux valeurs maxima décrites pour ces 2 acides dans le sang normal.

Discussion de la technique.

Afin que la modification de densité optique après addition de l'enzyme soit d'une amplitude suffisante pour permettre des lectures précises, nous avons été amenés, compte tenu de la faible concentration de l'acide malique plasmatique, à utiliser un volume de défécat (2,5 ml) très supérieur à celui auquel on a habituellement recours pour les dosages enzymatiques reposant sur le test optique de Warburg.

L'inconvénient qui en résulte est que la concentration de substances interférentes présentes dans le plasma se trouve également accrue.

C'est ainsi qu'après l'addition du tampon glycocolle/sulfate d'hydrazine/soude, il se produit une baisse progressive de la densité optique, suivie, après 15 min environ, d'une stabilisation de celle-ci. Cette baisse, vraisemblablement due à une interaction entre l'hydrazine et certains constituants du défécat sanguin, nous a conduits à attendre 15 min entre l'addition du tampon et celle de NAD^+.

Les modifications de la densité optique consécutives à l'addition de NAD^+ consistent en une augmentation immédiate, suivie d'une élévation plus discrète, mais progressive et d'importance variable selon les

défécats. Elles sont vraisemblablement dues à la formation non enzymatique d'un complexe d'addition entre le NAD^+ et le réactif du groupement carbonyle [6, 10].

En recherchant les facteurs permettant de réduire au minimum ces modifications de la densité optique survenant avant l'addition de malicodéshydrogénase, nous avons été amenés à diminuer considérablement la concentration en sulfate d'hydrazine par rapport à celle préconisée par HOHORST et coll. [4]. Dans ces conditions, les variations de la densité optique restent minimes ; nous éliminons néanmoins leur influence en utilisant comme témoin une cuve contenant tous les réactifs, sauf l'enzyme. Il faut signaler que ces variations de la densité optique ne peuvent être supprimées ni par la substitution du tampon As_2O_3/NaOH [6] au tampon glycocolle/soude, ni par adjonction d'acide éthylène-diamine-tétraacétique à la concentration finale de 0,75 µMole/ml.

La déshydrogénation de l'acide malique dans les conditions décrites est relativement lente, mais ne s'accompagne pas d'une altération de l'enzyme, comme le prouve la déshydrogénation complète d'une quantité supplémentaire d'acide L-malique ajoutée à la fin de la réaction. Nous avons recherché s'il était possible d'accélérer cette réaction en augmentant les quantités d'enzyme et de NAD^+. Il s'est avéré ainsi que la vitesse de la réaction n'est pas sensiblement modifiée en augmentant fortement la quantité d'enzyme (jusqu'à 375 unités). L'accroissement de la concentration en NAD^+ accélère, par contre, la réaction, mais accentue également les réactions secondaires dues aux substances interférentes, ce qui nous a conduits à ne pas dépasser la quantité de NAD^+ indiquée dans la description de la technique (3 µMoles).

En ce qui concerne la détermination de la fin de la réaction, nous estimons, comme indiqué plus haut, la déshydrogénation de l'acide L-malique terminée lorsque la différence de densité optique entre les 2 cuves reste stable pendant 20 min. On observe, dans certains cas, une diminution ultérieure de la différence entre les 2 cuves, la densité optique de la cuve-essai tendant à rejoindre celle de la cuve-témoin. HOHORST [11] a décrit, lors du dosage de l'acide lactique, un phénomène analogue, qu'il attribue à une réoxydation partielle du $NADH_2$, aboutissant à la formation d'un composé non identifié, ayant un maximum d'absorption à 400 mµ. Cette réoxydation peut être prévenue soit en augmentant la concentration en enzyme, soit en utilisant un dispositif de dosage à l'abri de l'air. De telles modalités techniques sont cependant généralement inutiles pour le dosage de l'acide L-malique sanguin, puisque ce n'est que dans des cas exceptionnels que nous n'avons pas observé un parallélisme entre les densités optiques de la cuve-essai et de la cuve-témoin pendant un temps suffisamment long pour permettre de fixer le moment de la fin de la réaction.

Comparaison avec la méthode fluorimétrique.

Nous avons déterminé, parallèlement aux dosages enzymatiques, l'acide malique sanguin à l'aide d'un photofluorimètre Photovolt par la technique de HUMMEL [1] modifiée seulement en ce qui concerne le volume de la prise d'essai (1 ml au lieu de 0,2 ml).

RÉSULTATS.

Le taux de l'*acide* L-*malique du sang total humain* trouvé chez 13 sujets sains à jeun est de 1,173 ± 0,270 µMoles/100 ml (moyenne arithmétique $\pm \frac{2\sigma}{\sqrt{N}}$) par la méthode enzymatique, alors que le résultat des dosages effectués parallèlement par la technique de HUMMEL [1] est de 1,93 ± 0,20 µMoles/100 ml.

Il apparaît donc que les valeurs obtenues à l'aide de la méthode enzymatique décrite sont notablement plus faibles que celles de la technique fluorimétrique, témoignant de la meilleure spécificité de la première.

Le taux de l'*acide malique plasmatique* dosé par la méthode enzymatique chez 40 sujets sains à jeun est de 0,413 ± 0,035 µMoles/100 ml. Nous n'avons pas constaté de différences significatives selon le sexe ou l'âge.

Dans le cas du plasma, comme dans celui du sang total, les résultats obtenus parallèlement par la méthode fluorimétrique sont notablement plus élevés (0,585 ± 0,048 µMoles/100 ml).

La constatation d'un taux d'acide L-malique beaucoup plus faible dans le plasma que dans le sang total nous a conduits à pratiquer le dosage enzymatique de cet acide dans les *hématies*. Nous y avons trouvé une concentration de 2,30 ± 0,55 µMoles/100 ml (moyenne de 8 déterminations).

Les taux moyens trouvés respectivement dans le sang total, le plasma et les hématies apparaissent ainsi concordants, compte tenu d'une valeur normale de l'hématocrite d'environ 40 p. 100.

DISCUSSION.

La concentration normale de l'acide L-malique dans le sang total humain (en moyenne 1,173 µMoles/100 ml) est beaucoup plus faible que celle des acides pyruvique (8,75 µMoles/100 ml selon STROHMEYER et coll. [12]) et citrique (9,6 µMoles/100 ml selon WOLCOTT et BOYER [13]). Elle est, par contre, du même ordre de grandeur que celle de l'acide α-cétoglutarique (0,96 µMoles/100 ml d'après STROHMEYER et coll. [12]). Elle est également voisine du taux normal trouvé par

GRÜNDIG [14] pour l'acide oxaloacétique (1,36 µMoles/100 ml) ; pour ce dernier acide, cependant, les résultats de KELLER et DENZ (2,86 µMoles/100 ml) [15] sont notablement plus élevés.

La répartition de l'acide malique entre plasma et hématies apparaît remarquable, puisque les hématies humaines sont beaucoup plus riches en cet acide que le plasma, alors qu'elles sont plus pauvres en acide citrique [16] et que les concentrations globulaires et plasmatiques sont sensiblement identiques dans le cas des acides pyruvique et α-cétoglutarique, comme en témoigne la confrontation des résultats concernant le sang total d'une part, le plasma ou le sérum de l'autre [12, 17, 18].

La richesse relative des hématies humaines en acide L-malique pose le problème de l'origine de cet acide.

Les hématies matures des mammifères ne possèdent pas de cycle tricarboxylique fonctionnel [19, 20]. La maturation du réticulocyte en normocyte s'accompagne, en effet, de la disparition presque totale de l'activité de la citrate hydro-lyase (4.2.1.3 ; aconitase) et de la succinate : (accepteur) oxydoréductase (1.3.99.1 ; succinodéshydrogénase) [21]. En l'absence de cycle citrique actif dans les normocytes, le métabolisme du glucose par la voie de la glycolyse y conduit à une accumulation de pyruvate [22] et de lactate [23, 24].

Le malate se forme vraisemblablement à partir de ces produits terminaux de la glycolyse. RUBINSTEIN et DENSTEDT [24] n'ayant pu mettre en évidence de L-malate : NADP oxydoréductase (décarboxylante) (1.1.1.40) dans les hématies, il est probable que le malate s'y forme, grâce à une GTP : oxaloacétate carboxy-lyase (4.1.1.32), par carboxylation du phospho-énol-pyruvate en oxaloacétate, suivie de la réduction de l'oxaloacétate en malate. Cette dernière s'effectue grâce à la L-malate : NAD oxydoréductase (1.1.1.37 ; malicodéshydrogénase), dont la persistance dans les globules matures des mammifères a été vérifiée par de nombreux auteurs [21].

L'augmentation de la concentration intra-érythrocytaire du malate, que nous avons constatée, au cours d'expériences préliminaires, en incubant des hématies humaines avec du glucose, est en faveur d'une telle synthèse endo-globulaire du malate à partir d'intermédiaires formés au cours de la glycolyse. Cette synthèse représenterait l'inverse de la transformation du malate en lactate, transformation qui a été prouvée dans les hématies de mammifères par SPICER et CLARK [25].

Le rôle de la synthèse endo-globulaire de malate paraît double. D'une part la carboxylation du phospho-énol-pyruvate en oxaloacétate nécessitant du GTP (ou de l'ITP), qui se forme lui-même à partir de l'ATP grâce à l'ATP : nucléoside-diphosphate phosphotransférase (2.7.4.6), consommerait de l'ATP, dont l'hématie adulte produit un excès [20]. D'autre part la réduction de l'oxaloacétate en malate, s'accompagnant d'une réoxydation du $NADH_2$ produit au cours de la

déshydrogénation du 3-phospho-glycéraldéhyde, permettrait, parallèlement à la réduction du pyruvate en lactate, d'éviter une accumulation de $NADH_2$.

Nous remercions vivement l'Institut National d'Hygiène (Directeur : Prof. L. Bugnard), qui a subventionné ce travail.

Résumé.

1) Les auteurs décrivent une technique enzymatique de dosage de l'acide L-malique sanguin et en comparent les résultats à ceux de la méthode de détermination fluorimétrique de Hummel, dont la spécificité est moindre.

2) Chez l'homme normal, la concentration de l'acide L-malique dans le sang total (1,173 ± 0,270 µMoles/100 ml) est du même ordre de grandeur que celle de l'acide α-cétoglutarique.

3) La répartition de l'acide L-malique entre plasma et hématies est remarquable, les globules étant beaucoup plus riches (2,30 ± 0,55 µMoles/100 ml) que le plasma (0,413 ± 0,035 µMoles/100 ml).

L'acide L-malique intra-globulaire, dont la concentration augmente lorsque l'on incube des hématies avec du glucose, pourrait se former par carboxylation du phospho-énol-pyruvate en oxaloacétate, suivie de la réduction de ce dernier en L-malate. Cette synthèse contribuerait à éviter l'accumulation intra-globulaire d'ATP et de $NADH_2$ produits en excès au cours de la glycolyse.

FOOT

Summary.

1. The authors describe an enzymatic technique for the determination of L-malic acid in blood and compare its results with those obtained by Hummel's fluorimetric determination, which is less specific.

2. In the normal human, the concentration of L-malic acid in total blood (1,173 ± 0,270 µmoles/100 ml) is of the same order of magnitude as that of α-ketoglutaric acid.

3. The distribution of L-malic acid between plasma and erythrocytes is remarkable : erythrocytes are much richer (2,30 ± 0,55 µmoles/100 ml) than plasma (0,413 ± 0,035 µmoles/100 ml).

Intracorpuscular L-malic acid, whose concentration increases when the erythrocytes are incubated with glucose, might be formed by carboxylation of phospho-enol-pyruvate to oxaloacetate, followed by reduction of the latter to L-malate. This synthesis would help to avoid accumulation in the corpuscules of ATP and $NADH_2$ produced in excess in the course of glycolysis.

Zusammenfassung.

1. Die Verfasser beschreiben ein enzymatisches Bestimmungsverfahren der Blut-L-Apfelsäure und vergleichen die Ergebnisse mit denen der fluorometrischen Bestimmungsmethode von Hummel, die weniger spezifisch ist.

2. Beim normalen Menschen ist die Konzentration der L-Apfelsäure im totalen Blut (1,173 ± 0,270 µMoles/100 ml) von derselben Grössenordnung wie diejenige der α-Ketoglutarsäure.

3. Die Verteilung der L-Apfelsäure zwischen Plasma und roten Blutkörperchen ist merkwürdig, denn die Blutkörperchen sind viel reicher (2,30 ± 0,55 µMoles/100 ml) als das Plasma (0,413 ± 0,035 µMoles/100 ml).

Die Blutkörperchen-L-Apfelsäure, deren Konzentration zunimmt, wenn man die roten Blutkörperchen mit Glucose inkubiert, könnte sich folgenderweise bilden : Carboxylation des Phosphoenolpyruvats zu Oxalessigsäure und Reduzierung der letzteren zu L-Malat. Diese Synthese würde dazu beitragen die Anhäufung des Blutkörperchen-ATP und -$NADH_2$, die im Laufe der Glycolyse erzeugt werden, zu vermeiden.

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THE FLUOROMETRIC DETERMINATION OF MALIC ACID*

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A sensitive and specific chemical method for the estimation of malic acid in biological materials has long been desired. The condensation of malic acid with orcinol in the presence of concentrated sulfuric acid to form the highly fluorescent homoumbelliferone (7-hydroxy-5-methylcoumarin) was reported years ago (1). Although this reaction is relatively specific for malic acid, the quantitative precipitation of calcium malate by alcohol as a means of fractionation from biological materials gives it additional specificity. If, in conjunction with this treatment, a small amount of 2,4-dinitrophenylhydrazine is added, most of the simple carbohydrate impurities which give an interfering amber color with orcinol become soluble in alcohol and thus may be removed.

Procedure

The solution to be analyzed is deproteinized with trichloroacetic acid of such a concentration that the final acidity is 1 N. Aliquots of the filtrate (containing 0.1 to 1.0 γ of malic acid) are transferred to 18 × 100 mm. test-tubes and are diluted to 1 ml. with 1 N HCl. To each tube are added with shaking 0.1 ml. of 0.1 per cent 2,4-dinitrophenylhydrazine in 2 N HCl and 0.5 ml. of 10 per cent $CaCl_2$ and, after 30 minutes, 0.3 ml. of 5 N NH_4OH and 6 ml. of absolute ethanol. After 12 hours at room temperature, the precipitation is complete. The tubes are strongly centrifuged and the supernatant is carefully poured off and discarded. To remove traces of moisture, the tubes are dried in the oven at 105° for 15 minutes.

A stock orcinol solution containing 80 mg. of orcinol (twice recrystallized from benzene) in 100 ml. of 12.5 per cent H_2SO_4 is prepared. This solution is stable for several months if stored in the cold in a brown bottle. To each tube, 3 ml. of orcinol-sulfuric acid mixture are added (8 ml. of stock orcinol solution diluted to 100 ml. with concentrated sulfuric acid of highest purity). The contents are mixed with bulb stirring rods (2).

The tubes are heated to 100° for 10 minutes, cooled under the tap, and diluted to 10 ml. with concentrated sulfuric acid. The blue fluorescence

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is measured in a Coleman photofluorometer against blank and standard tubes treated in a similar manner. An almost linear fluorescence curve is obtained as shown in Fig. 1. The fluorometric attachment to the Beck-

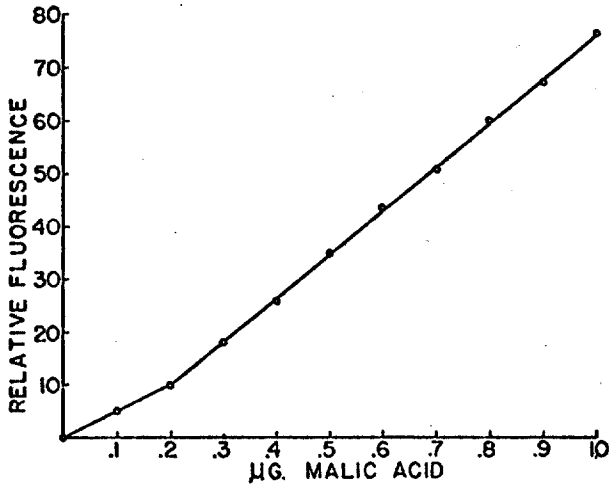

FIG. 1. The linear relation between the amount of malic acid assayed and the relative fluorescence produced.

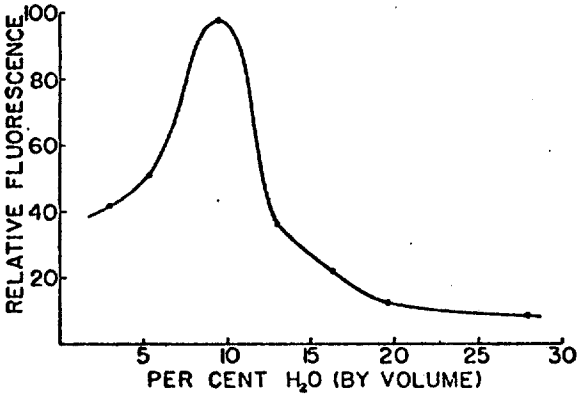

FIG. 2. The effect of different amounts of water in the orcinol-sulfuric acid mixture on the relative fluorescence produced by 1 γ of malic acid.

man spectrophotometer can also be used with somewhat greater concentrations of malic acid. The maximum fluorescence produced by 1 γ of malic acid is approximately equivalent to that given by 9 γ of quinine sulfate in 10 ml. of 0.1 N H_2SO_4. As shown in Fig. 2, the amount of water present during the condensation reaction is critical; less than 6 or more

ɔrometer against blank and standar
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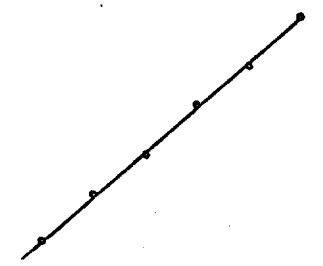

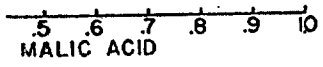

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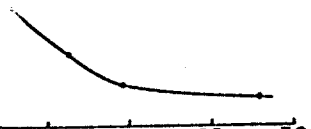

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ɜaction is critical; less than 6 or mor

than 12 per cent total water (by volume) in the sulfuric acid markedly reduces the fluorescence. It is therefore advisable to determine the water content of each batch of sulfuric acid.

TABLE I

Effect of Exercise on Content of Malic and Lactic Acids in Human Blood

Blood from finger-tips, 0.2 ml., was deproteinized with 6 ml. of 1 N trichloroacetic acid and centrifuged, and 1 ml. of the supernatant was treated as described in the text.

Subject No.	Malic acid	Lactic acid	Malic acid		Per cent recovery
			Added	Recovered	
		At rest			
	mg. per cent	*mg. per cent*	γ	γ	
I	0.75	11			
II	0.75	15			
III	0.63	15			
IV	0.62	45			
V	0.62	35			
VI	0.36	17			
VII	0.42	18	0.50	0.50	100
VIII	0.28	11			
IX	0.39	5	0.81	0.81	100
X	0.24	15	0.81	0.83	102
XI	0.36	14	0.81	0.80	99
XII	0.24	20	0.85	0.86	101
XIII	0.33	31	0.85	0.81	95
Average.....	0.46	19			
		After exercise			
IV	0.72	75			
V	0.59	85			
VI	0.51	53			
VII	0.37	86	0.50	0.50	100
XII	0.27	82	0.85	0.81	95
XIII	0.24	80	0.85	0.81	95
Average.....	0.45	77			

The following materials do not interfere at concentrations of 100 γ per tube: glucose, fructose, pyruvate, lactate, oxalacetate, isocitrate, aconitate, citrate, α-ketoglutarate, succinate, fumarate, β-hydroxybutyrate, acetoacetate, butyrate, tartrate, malonate, urate, glycerophosphate, aspartate, glutamate, alanine, and creatine. Samples of maleic acid tested gave

variable intensities of fluorescence, possibly due to contamination by malic acid. Fructose-1,6-diphosphate and glucose-6-phosphate do interfere since they are quantitatively precipitated by alcohol and produce both a yellow color and a blue fluorescence with the orcinol-sulfuric acid reagent. They may be easily removed from the sample by preliminary hydrolysis in 0.2 N NaOH for 10 minutes at 100°. Glycogen, which also interferes, may be removed by acid hydrolysis.

Malic Acid Content of Blood—As tested by this method, the levels of malic acid in finger-tip blood from normal male subjects were less than 1 mg. per cent, as shown in Table I, and did not change after exercise sufficiently vigorous to raise the blood lactic acid level to 3 or 4 times above the normal, as determined by the method of Barker and Summerson (3).

SUMMARY

A simple and sensitive fluorometric method for the determination of malic acid is described. The malic acid content of whole blood is low and does not change as a result of muscular activity.

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Apparatus

Procedure

amine hydrochloride solution (reagent *5*).
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st blank containing *n*-hexanol (reagent *8*).

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e f he copper procedure will be made.
ected in biological materials do not inter-
e present should be kept at a minimum. If
erly, copper-binding anions such as EDTA
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(~1 μg Cu) and 2 mg of organic matter.
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h). Five mμg atoms of Cu gives a net ab-
.115 under the conditions described. Since
is limited and therefore the quantity that
sorbance should not exceed 0.4. Absorbance
rtional below this value.
the procedure for copper and their pos-
those discussed above for iron.

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Rapid Separation of Some Common Intermediates of Microbial Metabolism by Thin-Layer Chromatography[1]

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Glyoxylic acid metabolism in bacteria has been the subject of extensive research in recent years and has been reviewed by Rabin *et al.* (1). Although glyoxylate can react enzymically with various compounds to form several important metabolic products (2–10), our laboratory has been concerned mainly with: (*a*) the condensation with acetyl-CoA to form malic acid (2); (*b*) the condensation with propionyl-CoA to form α-hydroxyglutaric acid (3); and (*c*) the reduction to glycolic acid (10). Although the fate of glycolic acid is unknown in bacteria (10), malic acid can be dissimilated by means of the tricarboxylic acid (TCA) cycle, and α-hydroxyglutaric acid is further metabolized either via citramalic (α-methylmalic) acid or succinic acid (11).

Studies have been initiated to determine the fate of glyoxylic acid in bacteria and fungi using the technique of pulse labeling with C^{14}-sodium glyoxylate. It was necessary, therefore, to develop a chromatographic technique that achieves a rapid and complete separation of glyoxylate from glycolate, malate, succinate, fumarate, α-ketoglutarate, α-hydroxyglutarate, and citramalate. Since α-hydroxyglutarate can lactonize under anhydrous conditions (12), this compound must also be separated. The method presented here permits the resolution of these nine intermediates associated with glyoxylic acid metabolism.

MATERIALS AND METHODS

Gelman ITLC, type SG chromatography medium (Gelman Instrument Co., Ann Arbor, Michigan) was used for the thin-layer chromatographic

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separation of the acids. The medium consists of glass microfiber support sheets which are impregnated with silica gel as the adsorbent. The sheets (20 × 20 cm) were loaded with 2.5 μl of each compound (0.3% w/v aqueous solutions) or with 20 μl of a standard mixture of these acids. α-Hydroxyglutaryl lactone was applied from a benzene solution.

Development was two-dimensional using the following solvent mixtures: (I) petroleum ether (b.p. 30–60°C), anhydrous diethyl ether, and formic acid (28/12/1), and (II) chloroform, methanol, and formic acid (80/1/1). All were Mallinckrodt products. The development chamber was rectangular with the following dimensions: 26 × 26 × 7 cm. The solvent fronts were allowed to ascend 15 cm, the sheets air-dried in a hood for 5 min, and the spots visualized by spraying with 0.04% bromphenol blue (ethanolic).

Samples to be measured for radioactivity were placed in 10 ml of 0.04% 2,5-bis-[2-(5-*tert*-butylbenzoxazolyl)]-thiophene (Packard Instrument Co.) made up in toluene absolute ethanol (2/1). These were counted in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Table 1 lists the R_f values obtained for each compound in the two solvent systems. Figure 1 demonstrates the resolution obtained by two-dimensional chromatography employing, first, solvent mixture I, followed by solvent mixture II. It is apparent that complete resolution of the nine acids is achieved.

Of further interest is the rapidity of the entire procedure. Each dimension required only 20–25 min for development and a sample can be resolved, as in Figure 1, in less than 1 hr. Several samples, therefore, can

TABLE 1
Separation of Intermediates Related to Glyoxylate Metabolism

Compound	$R_f \times 100$	
	Solvent I[a]	Solvent II[b]
Glyoxylate	17	55
Glycolate	59	50
α-Hydroxyglutarate	25	17
α-Hydroxyglutaryl lactone	33	73
Citramalate	41	21
Malate	17	11
α-Ketoglutarate	45	35
Succinate	80	65
Fumarate	97	70

[a] Petroleum ether (b.p. 30–60°C), anhydrous diethyl ether, and formic acid (28/12/1).
[b] Chloroform, methanol, and formic acid (80/1/1).

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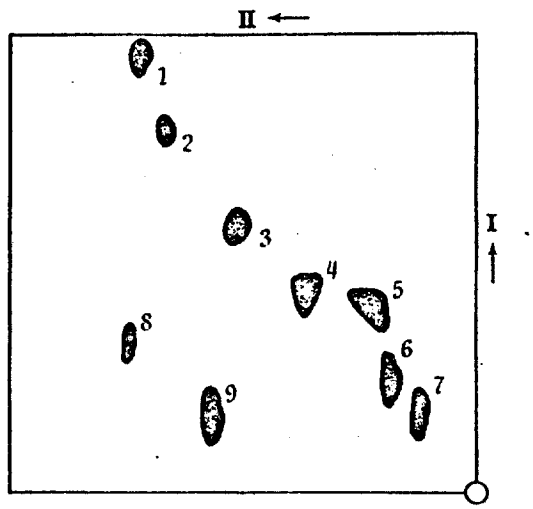

FIG. 1. Thin-layer chromatogram of intermediates associated with glyoxylic acid metabolism. The acids are numbered as follows: (1) fumarate, (2) succinate, (3) glycolate, (4) α-ketoglutarate, (5) citramalate, (6) α-hydroxyglutarate, (7), malate, (8) α-hydroxyglutaryl lactone, and (9) glyoxylate. The origin is indicated by the circle at the baseline. The solvent systems employed were: (I) petroleum ether (b.p. 30–60°C), anhydrous ether, and formic acid (28/12/1) and (II) chloroform, methanol, and formic acid (80/1/1).

be run in a short time. Myers and Huang (13) reported a total elapsed time of 7 hr for the two-dimensional separation of TCA cycle intermediates using conventional thin-layer chromatography with a cellulose adsorbent on glass plates.

In addition to excellent resolution and speed, there is another advantage to the method described here. The sheets, once developed and sprayed to visualize the spots, can be cut quite easily and the spots measured directly for radioactivity. This technique has been applied with success to prove the quantitative conversion of 5-C^{14}-α-hydroxyglutaric acid to its lactone (12). Background emission is negligible and accurate estimates of radioactivity can be obtained after correcting for quenching caused by the spray reagent. The radioactive spots are easily handled without damaging the surface layer.

SUMMARY

A method is presented which permits the complete and rapid chromatographic resolution of nine biologically important carboxylic acids. The technique utilizes two-dimensional thin-layer chromatography and separates glyoxylate, glycolate, α-ketoglutarate, malate, succinate, fumarate, citramalate, α-hydroxyglutarate, and α-hydroxyglutaryl lactone. Some of

these acids are intermediates of the TCA cycle and can also be resolved by the TLC procedure recently presented by Myers and Huang (13). We feel, however, that the speed, convenience, and reproducibility of the procedure described here offers many advantages over previously described methods.

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Two-way separation of carboxylic acids by thin layer electrophoresis and chromatography

In the course of investigations carried out in this laboratory, a method was required for the analysis of a mixture of carboxylic acids. Although some work has been reported on thin layer chromatographic separations using silica gel[1,2] and cellulose[3,4] as the stationary phases, with either an acid[1-4] or an alkaline[3] mobile phase, there does not appear to be any reference to a two-dimensional system including an electrophoretic separation. This paper describes a method which allows the simultaneous separation of at least 15 carboxylic acids, in a two-dimensional system within 5 h. The separation is started by electrophoresis in formic acid buffer, in the first dimension, followed by chromatography in isoamyl alcohol saturated with formic acid in the second.

Reagents

Cellulose powder MN 300 Macherey, Nagel & Co was used. Formic acid and isoamyl alcohol were of analytical grade from British Drug Houses; 2,7-dichlorofluorescein was from Merck, Darmstadt. The carboxylic acids were of analytical grade either from British Drug Houses or from Merck.

Apparatus

The cellulose layers were prepared with Desaga equipment (Desaga, Heidelberg) on 20 × 20 cm glass plates. Electrophoresis was carried out with a Desaga electrophoresis chamber with an aluminium alloy cooling plate, cooled by tap water, and a 1000 V Pleuger power supply CVC D, Belgium, with a built-in 60 mA ammeter. Desaga tanks were used for chromatography.

Experimental

The thin-layer plates were prepared with the Desaga equipment, using 17.5 g cellulose powder per 100 ml water. The plates were left to dry at room temperature for 24 h, and before use, they were prepared as shown in Fig. 1. The carboxylic acid mixture was applied with a Carlsberg pipette for volumes up to 5 μl. Larger samples were applied with an automatically driven Hamilton syringe, under a stream of cold air. The plates were cooled to about 4° and sprayed with a cold 0.2 M formic acid buffer, pH 2.5 (ammonia was used to raise the pH of the pure formic acid solution before adjusting to final volume). To avoid diffusion of the sample, a small (5 × 5 mm) piece of parafilm was placed over the application point during the spraying. The plates should be sprayed carefully in order that they do not become too wet, and spraying should be stopped when the surface appears shiny, indicating that the layer is moisture saturated[6]. Excess buffer on the glass edges was removed, and the plate was placed in the Desaga electrophoresis chamber. Two Whatman No. 1 paper wicks (10 × 20 cm) were used to connect the plate with the buffer troughs. In most runs a potential of 1000 V (50 V/cm) was applied for 30 min. Under these conditions the current only increased from an initial value of 20 mA to a final one of 25 mA.

After the electrophoresis the plates were removed and dried in a stream of cold air for about 30 min. During this time the buffer evaporated. Before chromatography, the edges of the plates (5 mm) which had been overlapped by paper wicks

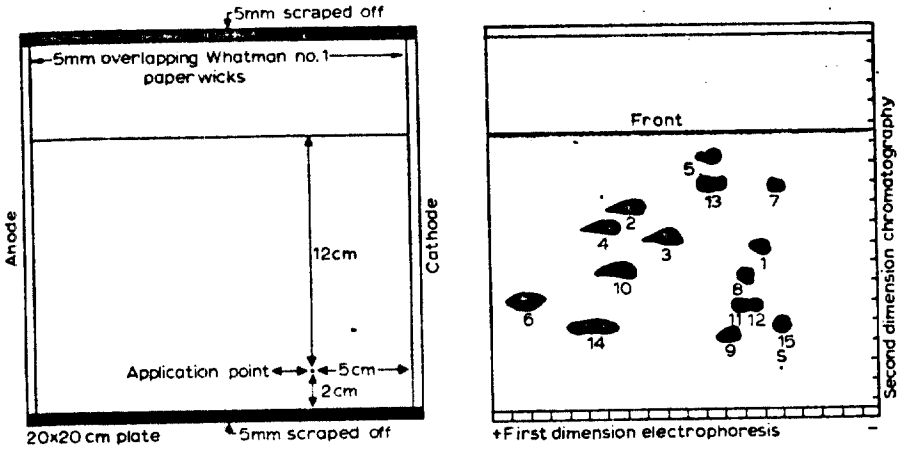

Fig. 1. Schematic diagram of a thin-layer plate showing the treatment of the plates prior to electrophoresis and chromatography. The line drawn at 12 cm was used to visualise the solvent front position during the chromatographic step.

Fig. 2. Thin-layer chromatogram of a two-dimensional separation of carboxylic acids. Cellulose layer 375 μ. An aqueous solution containing 10 μg of each acid was applied to the starting point (S). 1 = Glycolic; 2 = fluoroacetic; 3 = malonic; 4 = pyruvic; 5 = fumaric; 6 = maleic; 7 = succinic; 8 = malic; 9 = tartaric; 10 = α-ketoglutaric; 11 = citric; 12 = isocitric; 13 = *trans*-aconitic; 14 = *cis*-aconitic; 15 = ascorbic acid. For details see Experimental.

were scraped off. Chromatography was carried out in the second dimension with isoamyl alcohol–5*M* formic acid (2:1). A filter paper strip (15 × 20 cm), wetted in the lower acid layer, was used to line the tank in such a way that it did not touch the solvent lying in the bottom. A tank thus prepared could be used for at least three days. In order to obtain sharp spots it was found necessary to equilibrate the plates for about 30 min. The chromatographic separation was carried out using the upper phase of the above mixture as solvent, with 2,7-dichlorofluorescein (5 mg per 100 ml upper phase) dissolved in it[5]. This addition to the solvent allowed the detection of the carboxylic acids on the plates without spraying. The chromatography was stopped when the solvent front had reached 12 cm from the starting point. This took about 2½ h. After chromatography the plates were dried under a stream of cold air for about 30 min until all the solvent had been removed. This was necessary in order to increase the pH difference between the spots and the plate. On drying the carboxylic acid spots could be detected by observation under ultraviolet light (254 nm). Fig. 2 shows a typical chromatogram with the separated acids. For documentation the plates were photographed (Fig. 3) in ultraviolet light (254 nm) with a yellow filter (Kodak wratten 12) covering the camera lens.

Results and discussion

The method described allows the separation of a mixture of at least 15 carboxylic acids as shown in Fig. 2. With pure samples it was possible to start with the chromatographic separation, however with impure samples it was found convenient to start with electrophoresis as this step not only desalted the sample, but also separated the carboxylic acids from amino acids that could be present in the sample. Furthermore the use of a volatile buffer allows chromatography in any solvent system.

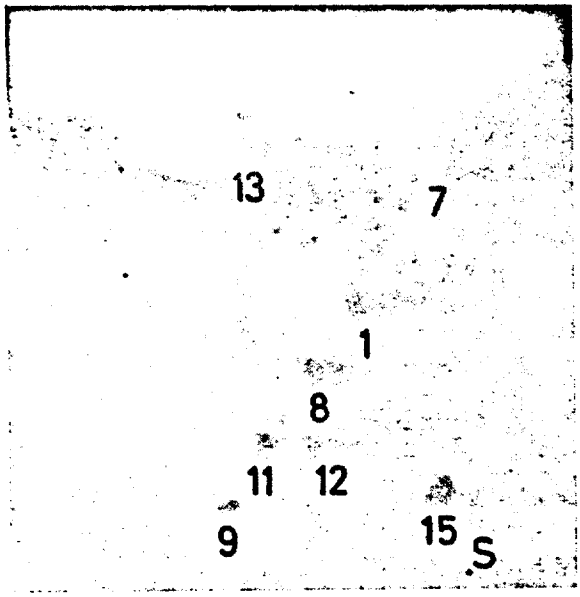

Fig. 3. Ultraviolet photograph of thin-layer chromatogram of a mixture of carboxylic acids, indicated by the same numbers as in Fig. 2. The electrophoresis has been carried out for 60 min to separate citric (11) and isocitric acid (12).

Some of the factors which affected the separation were:

Nature of the supporting material. Cellulose was preferred to silica gel, as with the latter too much heat was evolved on electrophoresis.

Plate thickness. It was found that 375 μ was the optimal thickness. Thinner plates could also be used with a lower capacity and reproducibility. On the other hand plates as thick as 500 μ could be used but the heat evolution was such that the power supply was easily overloaded.

pH. Similar separation patterns to the one presented were obtained with a lower pH, but the distances between the different spots were smaller. A higher pH than 2.5 resulted in increased heat evolution with no improvement in the resolution.

Buffer concentration. Lower concentrations than 0.2 M gave poorer separation, while higher concentrations resulted in increased heat evolution.

During the electrophoresis the acids migrated towards the anode, however owing to the endosmotic flow some acids appeared to remain stationary at the origin or even moved slightly towards the cathode. A longer run (60 min) resulted in the loss of maleic acid. On the other hand this permitted a separation of citric and isocitric acid (see Fig. 3). It should be mentioned that oxalic acid could not be detected by this method, as it formed very diffuse spots. It is important to take photographs within a few hours of development, since the spots weaken with time. BACHUR[8] has published a detection method based on the ability of acids to inhibit the browning of pyridine treated cellulose under ultraviolet light; this method could also be used.

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The detection of benzyloxycarbonyl-protected amino acid and peptide derivatives on thin-layer chromatograms

The application of thin-layer chromatography to peptide chemistry has been the subject of a recent monograph[1].

The classical benzyloxycarbonyl group, introduced by BERGMANN AND ZERVAS[2], is still the most common amino-protecting group used in the synthesis of peptides. We have for some time been investigating the possibility of detecting benzyloxycarbonyl-protected amino acid and peptide derivatives on chromatographic plates using ninhydrin. Our original plan was either to add a deblocking agent to the ninhydrin solution or to follow spraying with a deblocking reagent by treatment with ninhydrin. After inspection of the methods already developed for the removal of benzyloxycarbonyl groups in preparative work, it seemed possible to us that an acid, such as trifluoroacetic acid (WEYGAND AND STEGLICH[3]), would be worth testing. We chose for this purpose the less volatile trichloroacetic acid. In this way, by either using a ninhydrin solution in *n*-butanol containing 10 % trichloroacetic acid, followed by heating to 100°, or by spraying with a 10 % solution of trichloroacetic acid in glacial acetic acid, followed by heating to 100° and then spraying with ninhydrin, satisfactory spots could be obtained from some benzyloxycarbonyl compounds. However, a cleaner procedure, not involving the unpleasant trichloroacetic acid, seemed desirable.

Very recently WOLMAN AND KLAUSNER[4] published a procedure for the detection of *tert.*-butyloxycarbonyl derivatives on chromatograms, based on the sensitivity of these compounds to heat. Thus, after heating thin-layer chromatograms to 125–130° for 25 min, ninhydrin-positive spots were obtained.

We have now found that benzyloxycarbonyl compounds, too, are sensitive to heat. A somewhat higher temperature is needed for most benzyloxycarbonyl compounds than for *tert.*-butyloxycarbonyl compounds, although some can be detected

such as elymoclavine and agroclavine which, unlike those produced on unsupplemented media, are not derivatives of lysergic acid. Since all of the alkaloids isolated were labelled to approximately the same extent (see Table), a common biogenetic pathway is indicated. The high specific activities show that

Table

Specific Activity of Compounds Isolated from 35-*Day-Old Culture of* Claviceps purpurea (PRL 1578)

Compound	*Specific activity* (mC/*M*)
Ergometrinine	93
Ergocorninine	84
Ergotaminine	78
Ergosine	80
Ergosinine	97
Ergocryptine	71
Ergocryptinine	77
Agroclavine	72
Elymoclavine	64
Tryptophan from protein	57
Tryptophan from medium	133

they must be derived in part from the D-isomer. Partial racemisation of the amino acid may occur in the medium before incorporation but racemisation cannot be complete since the free tryptophan isolated at the end of the experiment had $[\alpha]_D^{20°}$ −28·8° and a specific activity higher than the value for total added tryptophan. This latter result is consistent with preferential utilisation of the less active L-isomer and also suggests a negative feedback mechanism in *C. purpurea* preventing endogenous tryptophan synthesis.[4] Although tryptophan was the only radioactive amino acid in the culture and in mycelial protein at the end of the experiment, some activity was found in an amphoteric, water-soluble, brown pigment, which appeared in both the free amino acid fraction and the alkaline protein hydrolysate.

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* N.R.C. contribution No. 5351.

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HIGH-VOLTAGE PAPER ELECTROPHORESIS OF ORGANIC ACIDS AND DETERMINATION OF MIGRATION RATES

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It was shown in a previous communication[1] that good separations of non-volatile organic acids in complex mixtures could be obtained within comparatively short periods of time by application of high potential gradients and a background electrolyte of pH 2·0. The usefulness of the method has since been extended to the lower fatty acids[2] and inorganic acids[3] by using an approx. 0·1 M ammonium carbonate solution of pH 8·9.

A systematic investigation into the migration rates of a great number of non-volatile organic acids over the pH range 1·5–11·5 has produced the information that, by choosing buffer or electrolyte solutions of three different pH values with concomitant variations in the migration rates, a far greater separation efficiency and reliability of identification could be obtained. The solutions found most suitable were:

(i) 0·75 M Formic acid solution of pH 2·0; (ii) 0·5 M Acetic acid solution adjusted with pyridine to pH 4·0; (iii) Approx. 0·1 M ammonium carbonate solution (7·9 gm./l.) of pH 8·9.

The migration rates were measured by comparing the rates with that of the chloride ion run on the same electrogram and thus under the same conditions, and with sucrose spots as markers for the electro-osmotic and hydrodynamic flow effects. The apparatus used was a larger version of the enclosed strip type with more efficient top and bottom aluminium cooling plates, polythene insulation and highly reproducible pneumatic pressure control,[1] which allowed the use of sheets of 12 in. × 22½ in. and thus the application of up to 10 spots at a time without mutual interference.

Although this type of apparatus greatly minimises evaporation and consequent influx of electrolyte solution converging from both ends towards the centre of the sheet, it was nevertheless found to occur to an extent which would make the results of measurements even of relative migration rates doubtful.

By interposing cellophane membranes between the ends of the sheet and the thick filter paper wads connecting it with the electrode vessels, utilising Weber's observation,[4] the influx was largely suppressed and the correction for electro-osmosis and minor possible effects made relatively simple, since there was only a uniform flow from the anode end towards the cathode end, as revealed by sucrose markers distributed evenly over the whole area of the sheet. The migration rate was measured as the distance of the centre of the spot from the starting line plus the shift of the relevant sucrose marker from the starting line, with all compounds migrating towards the anode. In the case of unduly elongated spots the distance to within 1·5 cm. from the leading edge was measured, which was assumed to be the centre of the spot. All electrophoretic experiments were carried out in quintuplicate and the quoted figures are the mean of the results which varied within 5%. The pressure applied was 1·5 lb/s.in. and the moisture content of the soaked and compressed sheet was found by weighing to be 145% (±3%) by weight, compared with dry paper.

The conditions chosen were as follows: (*a*) At pH 2·0, 100 V./cm., 6·5 m.amp./cm., 25 minutes, temp. of cooling water: 15·5°c.; (*b*) at pH 4·0, 100 V./cm., 9·6 m.amp/cm., 20 minutes, temp. of cooling water 16°c.; (*c*) at pH 8·9, 80 V./cm., 8 m.amp./

cm., 25 minutes, temp. of cooling water 8°c. Under these conditions the mobilities of Cl^- were $37{\cdot}1\times10^{-5}$ (cm.^{2} sec.$^{-1}$ V^{-1}) at pH 2·0, $36{\cdot}6\times10^{-5}$ at pH 4·0, and $29{\cdot}0\times10^{-5}$ at pH 8·9 (and lower temperature). This is still far below the mobility of Cl^- for free migration at infinite dilution, viz. $65{\cdot}4\times10^{-5}$ at 18°c.

The results are summarised in Tables I and II with column 5 containing the figures for dissociation constants, compiled from the literature,[5] to demonstrate the varying relationship between migration rates and dissociation constants, quite marked at pH 2·0, but only loose at higher pH values. This allows the separation of acids of similar mobilities by varying the pH of the electrolyte solutions, and forms the basis for two- or multi-dimensional paper electrophoresis, results of which will be reported elsewhere.[6]

Table I

Relative Migration Rates of Aliphatic Acids

Acid	M_{CL}* *at pH* 2·0	M_{CL} *at pH* 4·0	M_{CL} *at pH* 8·9	*Dissociation constant at* 25°c. $K\times10^5$
Cl^-	1·00	1·00	1·00	
Trichloroacetic	0·43	0·39	0·37	20000
Dichloroacetic	0·41	0·45	0·42	5000
Oxalic	0·41	0·48	0·70	6500
Meconic	0·40	0·42	0·27	
Maleic	0·28	0·45	0·59	1500
3-Phosphoglyceric	0·28	0·43	0·48	
Cis-Aconitic	0·23	0·38	0·63	
Oxalsuccinic	0·17	0·40	0·57	
α-Ketoglutaric	0·17	0·40	0·57	
Oxalacetic	0·16	0·34	0·48	
Pyruvic	0·16	0·48	0·50	300
Dihydroxytartaric	0·15	0·45	0·63	1200
Citraconic	0·13	0·35	0·53	348
α-Ketobutyric	0·13	0·28	0·42	
Malonic	0·10	0·44	0·63	140
Monochloroacetic	0·08	0·46	0·45	150
Fumaric	0·06	0·40	0·61	100
Tartronic	0·06	0·40	0·57	500
Trans-Aconitic	0·05	0·38	0·63	136
Tartaric	0·05	0·40	0·59	110
2-Ketogluconic	0·05	0·28	0·25	1570
Glyoxylic	0·04	0·39	0·57	50
Citric	0·04	0·30	0·45	87
Mesaconic	0·04	0·34	0·57	82
Galacturonic	0·03	0·22	0·22	
Malic	0·03	0·32	0·57	40
Dihydroxymaleic	0·03	0·38	0·66	
β-Hydroxybutyric	0·02	0·12	0·35	
Tricarballylic	0·02	0·23	0·59	92
Glycollic	0·02	0·31	0·48	15
Ascorbic	0·02	0·12	0·25	
Lactic	0·02	0·25	0·42	14
Adipic	0·02	0·10	0·48	3·7
Itaconic	0·02	0·23	0·57	15
Glutaric	0·02	0·13	0·53	4·5
Gluconic	0·02	0·20	0·25	
Laevulinic	0·02	0·09	0·35	
Acrylic	0·01	0·14	0·45	5·5
Succinic	0·01	0·18	0·57	6·6
Sorbic	0·01	0·05	0·32	1·7
Pimelic	0·01	0·09	0·45	3·1
Suberic	0·01	0·08	0·45	3·0
Sebacic	0·01	0·05	0·41	2·7

$$*M_{CL} = \frac{\text{Migration rate of acid}}{\text{Migration rate of chloride}}$$

Table II

Relative Migration Rates of Aromatic Acids

Acid	M_{CL}* *at pH* 2·0	M_{CL} *at pH* 4·0	M_{CL} *at pH* 8·9	*Dissociation constant at* 25°c $K\times10^5$
Cl^-	1·00	1·00	1·00	
Sulphosalicylic	0·35	0·41	0·53	
Picric	0·26	0·23	0·22	16000
3-Nitrosalicylic	0·19	0·21	0·24	1570
5-Nitrosalicylic	0·13	0·22	0·22	890
Phthalic	0·05	0·27	0·45	126
Salicylic	0·04	0·24	0·32	106
Sulphanilic	0·03	0·30	0·37	
Pyrrolidone-Carboxylic	0·03	0·31	0·35	
Syringic	0·01	0·03	0·22	
Benzoic	0·01	0·14	0·37	6·3
Tannic	0·01	0·03	0·29	

$$*M_{CL} = \frac{\text{Migration rate of acid}}{\text{Migration rate of chloride}}$$

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STRUCTURE OF THE TERPENOID GEIJERENE

By M. D. Sutherland

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The hydrocarbon geijerene, $C_{12}H_{18}$, was isolated by Penfold and Simonsen[1,2] from the leaves of *Geijera parviflora* Lindley and was shown to be a monocyclic triene. Degradative work[2] resulted merely in the isolation of formaldehyde and formic acid from the oxidation of geijerene, and a ketone, $C_{11}H_{20}O$, from tetrahydrogeijerene.

Pure geijerene[3] (I)$[\alpha]_D\pm0{\cdot}00°$ shows only end absorption at 220 mμ and absorption peaks at 1820, 1798, 1641, 1002, 911, 896, 784, 716, 705, 673 and 661 cm.$^{-1}$, suggesting the presence of a quaternary vinyl group, an *iso*propenyl group and a *cis* disubstituted double bond. Ozonization by Doeuvre's method yielded 2·0 moles of formaldehyde, and the *CMe* estimation (O_3/H_2CrO_4) 1·08 and 1·11 moles of volatile acid.

Dehydrogenation over *Pd/C* at 330° yielded only 1 : 4-dimethyl-azulene (II), identified by the ultraviolet and visible spectra, and by the melting points of the picrate (144°) and trinitrobenzolate (173°). Unidentified naphthalenes were also formed.

ANALYTICAL BIOCHEMISTRY 30, 32–39 (1969)

Separation and Quantitative Determination of Lactic, Pyruvic, Fumaric, Succinic, Malic, and Citric Acids by Gas Chromatography

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A simple and accurate method for the rountine quantitative analysis of organic acids is needed to study physiological and pathological processes in biological materials. In the past, gradient elution of silica columns, paper and thin-layer chromatography, and the analysis of precipitated lead acid salts were widely used (1–7). More recently, the gas-liquid chromatography of the volatile derivatives of organic acids has been studied.

The efficiencies of different esterification methods and the chromatographic separation of the resulting esters have been described (9–12). The molar response of the flame ionization detector to organic acid methyl esters has been studied (13). In the present study, six organic acids were esterified, chromatographed, and standard curves were established by relating ester peak heights to an internal standard. This gas chromatographic method was designed to separate and quantitate some representative types of organic acids while eliminating as much sample handling as possible.

METHODS AND MATERIALS[1]

Apparatus

A Varian Aerograph, model 1520B, gas chromatograph operated as a single column instrument with a flame ionization detector (FID) was used for this study.

[1] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

Column Preparation

A stainless-steel tube, 3 m long and 2.5 mm i.d., was filled with a 5% solution of dimethyldichlorosilane in toluene. After standing for 5 min, the tube was emptied and washed with methanol (MeOH) until the effluent was neutral to litmus (14).

The dried tube was packed with 5% diethylene glycol adipate (high-temperature stabilized, Analabs), on 100/120 mesh, A/W, DMCS, Chromosorb W. The liquid phase was applied using the method of Horning, Moscatelli, and Sweeley (15).

Chromatographic Conditions

The following parameters were used for this study:

Injector: 195°C.
Detector: 220°C.
Hydrogen: 20 ml/min.
Air: 350 ml/min.
Electrometer range: 10^{-11} A, full scale.
Carrier gas: Nitrogen 99.95% pure, oil-free, meets Federal Specification BB, 45 psig.

The linear flow velocity was set at 6.3 cm/sec at 60°C with lab gas as the unsorbed solute. The oven temperature was maintained at 60°C for 6 min following sample injection. The temperature was then programmed at 7°/min to 185°C and maintained at 185° until after the elution of trimethyl citrate.

Reagents

The reagents used were methanol (Baker, anhydrous), chloroform (Baker), boron trifluoride (Metheson Gas), lactic acid (Fisher, 87.2%), sodium pyruvate, disodium fumarate, succinic acid, malic acid, and citric acid (Calbiochem).

The commercial methyl esters of these organic acids were diluted in chloroform and chromatographed to establish their retention times as known standards. The purity of these esters was not determined. The esters were used only to establish retention times.

Esterification

Alcock's method was modified for this study (16). Volumetric pipets were used throughout the procedure. 100 mg of each acid, or an equivalent weight of acid salt, was weighed into a 100 ml volumetric flask. Then 1 ml of 6 *N* HCl was added and the flask was made to volume with 70% ethanol. The acid was used only to dissolve sodium salts in

ethanol. Aliquots of solution equal to 0.5, 1.0, 2.0, and 5.0 mg of acid were placed into 250 ml ₸ ball flasks and 1.0 ml of 2 *N* NH_4OH was added to each flask. The NH_4OH is used to make the ammonium salts of the acids and reduce their volatility. The flasks were dried at 20°C on a rotary evaporator at reduced pressure.

2 ml of a boron trifluoride/methanol solution (125 gm/liter) was pipetted into each flask and the mixture was held overnight (approximately 16 hr) at room temperature.

Extraction and Chromatography

4 ml of a 52% saturated $(NH_4)_2SO_4$ solution and 2.0 ml of $CHCl_3$ were pipetted into each flask and the contents mixed. In the extraction, $(NH_4)_2SO_4$ was added to the water to salt the esters out of the aqueous MeOH phase. This solution was found to be more effective than either a similarly saturated NaCl solution or water alone.

The proportions for the ammonium sulfate solution and chloroform were chosen to give the greatest concentration of esters in a final solution without an objectionable amount of MeOH carryover.

The contents of the flask were transferred to a separatory funnel by allowing the flask to drain for 30 sec. The separatory funnel was shaken for 1 min and the layers allowed to separate. The lower (chloroform) phase was drained into a small beaker and a 1.0 ml aliquot placed into a small screw-capped vial containing 100 mg of anhydrous Na_2SO_4. Then 0.1 ml of methyl laurate internal standard solution (2 ml methyl laurate diluted to 100 ml with $CHCl_3$) was added to the vial with a 0.25 ml syringe and 25 gage needle. The vial was covered with aluminum foil, and a Teflon-lined cap was screwed on tight. Samples were taken through the aluminum foil to avoid evaporation. Duplicate 1.0 μl injections were made with a Hamilton 10 μl syringe with a 1 in. needle. Sufficient $CHCl_3$ was left in the syringe to compensate for the needle dead space.

A test was made to determine the effect of temperature on ester extraction. After 20 min to achieve thermal equilibrium, extractions were made at 81°, 76°, and 68° F in controlled-temperature rooms. Over this range, the relative amounts of esters extracted was not affected by the temperature.

Quantitation

The ratio (R) of the acid ester peak height to the peak height of the internal standard was determined (14). The average R was plotted against the weight of the organic acid taken for esterification:

$$R = \frac{\text{ester pk. ht.} \times \text{attenuation}}{\text{standard pk. ht.} \times \text{attenuation}}$$

Peak height can be used as a measure of the quantity of the ester chromatographed if peaks are sharp, if the recorder is zeroed, and if a baseline at the proper attenuation is established before and after the peak.

RESULTS AND DISCUSSION

The wide range of organic acids found in biological materials necessitates the use of temperature-programmed gas chromatography. To avoid excessive high temperature bleed it was necessary to use a high-temperature stabilized liquid phase.

A rapidly eluting nontailing solvent is a necessity for this analysis. When MeOH was used as the injection solvent it had a broader solvent front than $CHCl_3$, tailed, and never reached a satisfactory baseline. In addition, MeOH injections resulted in a broad peak form beginning approximately 6 min after injection, corresponding to the initiation of the temperature program (Fig. 1, curve B). The identity of this "peak"

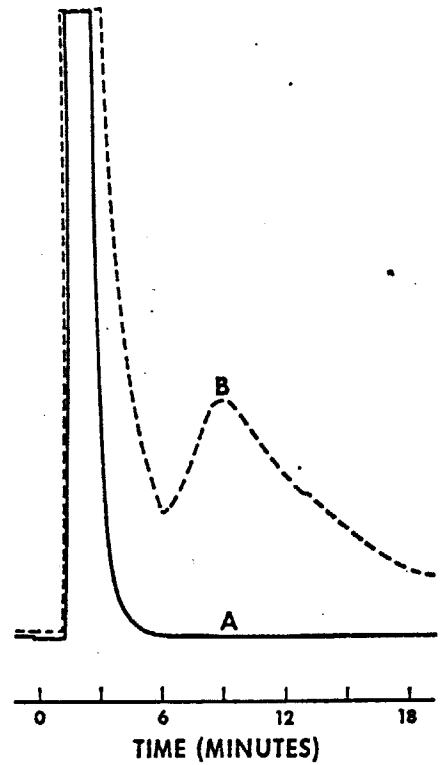

FIG. 1. (A) Chromatogram of 1.0 μl $CHCl_3$ injected at 60°C; temperature program initiated 6 min after injection. (B) 1.0 μl MeOH injected under the same conditions.

was not determined. The result of the remaining traces of MeOH in the $CHCl_3$ extract can be seen as a rounded hump under peak "a" in Figure 2.

Methanol is sufficiently hydrophilic to be retained in the aqueous phase when the solution is extracted with chloroform. The quantity of methanol carried over with the chloroform is inversely proportional to the amount of aqueous phase. Most of the esters are slightly soluble in water and freely soluble in chloroform, but each additional volume of water added reduces the amount of ester recovered in the extraction. Complete extraction of the esters, except methyl lactate, could be achieved by multiple chloroform extractions. This procedure would require evaporation of most of the chloroform without a corresponding loss of esters, quantitative transfer to a volumetric flask, and dilution to a volume of about 2 ml if any concentration advantage were to be obtained. The single extraction technique used avoids this additional sample handling and gives satisfactory results.

Chloroform was selected as the best solvent to extract the esters from the BF_3/MeOH esterification medium after testing CS_2, pentane, benzene, Freon 11, and ethyl ether. None of these solvents gave the combination of solvent properties, rapid elution, and water immiscibility of $CHCl_3$. Chloroform is not an ideal solvent. It is partially converted to HCl gas by the high temperatures of the flame ionization detector. The HCl will corrode some of the metal parts in the detector, eventually causing excessive noise or spiking. This problem was eliminated by placing a machined Teflon cap over the flame base assembly and allowing the quartz flame tip to protrude through a hole in the top of the cap.

Figure 2 is a typical chromatogram of the six organic acid esters. The small peak on the leading slope of peak "e" is an impurity in the methyl laurate and was constant for all injections. It was found that the retention time of methyl lactate decreased about 1 min as concentration increased from 0.5 to 5.0 mg of esterified acid. This phenomenon has been noted for another hydroxy compound (*n*-hexanol) chromatographed on open tubular nonpolar columns (17).

The major esterification product of pyruvic acid was methyl 2,2-dimethoxypropionate (MDMP). This product was previously identified (18). In the control solution of methyl esters, methyl pyruvate chromatographs as peak "a" (Fig. 2). In the esterified acid sample the MDMP chromatographs as peak "c" as shown in Figure 2. The identity of this product was determined by interpretation of the MS, IR, and NMR spectra after collection from a preparative GLC column.

In contrast to the results of other esterification methods, the use of BF_3/MeOH favors the formation of MDMP (11, 18). At the highest concentration used in the acid esterification, MDMP accounted for 99%

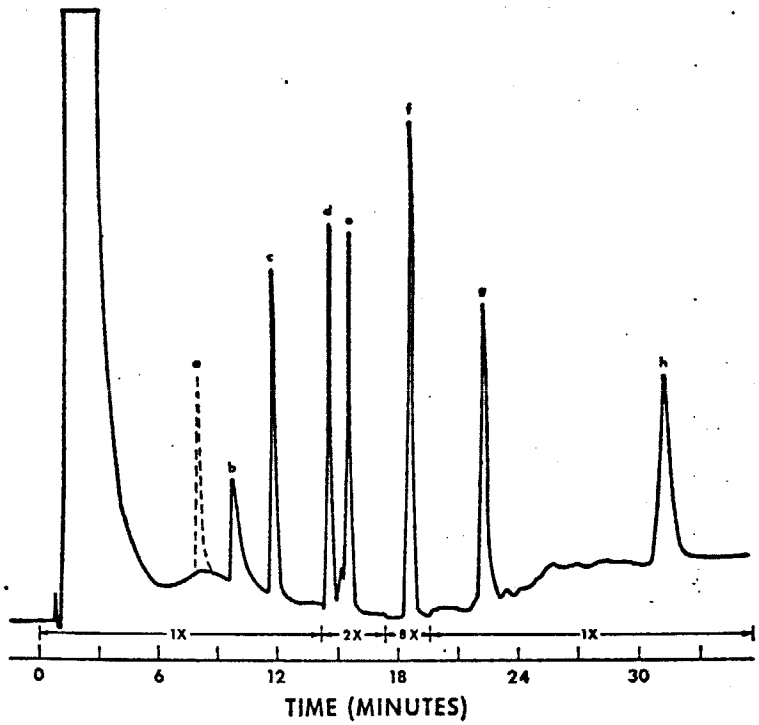

FIG. 2. Chromatogram of esterified and extracted organic acids: (a) methyl pyruvate (see text), (b) methyl lactate, (c) methyl 2,2-dimethoxypropionate, (d) dimethyl fumarate, (e) dimethyl succinate, (f) methyl laurate (internal standard), (g) dimethyl malate, and (h) trimethyl citrate.

of the pyruvic acid esterified and chromatographed. At lower concentrations the methyl pyruvate is too small to be measured.

The linear relationships between R value and the weight of organic acid taken for esterification are shown in Figure 3. The curve for MDMP has a negative intercept and can be used for the quantitation of pyruvic acid if the amount of MDMP injected is greater than 0.5 μg. Detection limits will depend on the esters, but all can be detected at the 0.2 μg injection level at the indicated instrument parameters.

In order to obtain satisfactory quantitative results with this procedure, the initial temperature and the temperature program must be duplicated exactly from run to run. Minor differences in initial temperature, or rate of temperature increase, will give variations in peak shape and reduce the precision. Duplicate injections should not differ by more than 3% of the average R.

Long periods of column inactivity resulted in excessive bleed at the upper limit of the program for the first one or two injections. This prob-

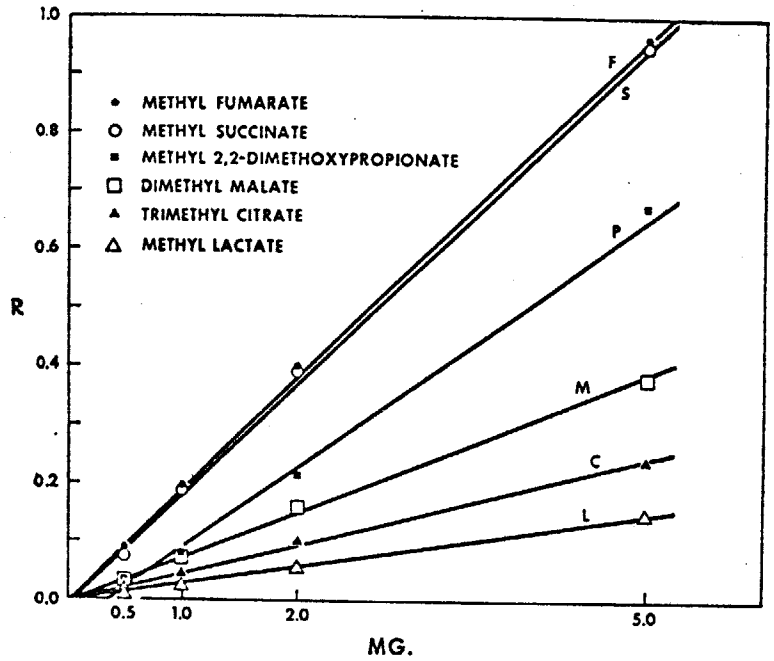

FIG. 3. Plot of R values vs. original weight of organic acid to be esterified.

lem was solved by raising the column temperature to 200°C for about 15 min at the beginning of the day and running one injection through the program before attempting quantitative work.

SUMMARY

A method for the esterification, extraction, and quantitative gas-liquid chromatography of lactic, pyruvic, fumaric, succinic, malic, and citric acids has been described. The effect of temperature variation on extraction is discussed and optimum conditions necessary for extraction are given. The quantitative conversion of pyruvic acid to methyl 2,2-dimethoxypropionate and its chromatographic characteristics are reported.

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[70] Fluorometric Assay of Malic Acid and Its α-Substituted Derivatives[1]

By MURRAY STRASSMAN and LOUIS CECI

Principle. This fluorometric assay is based on the degradation of substituted malic acids to β-keto acids, which condense with resorcinol to form derivatives of the highly fluorescent 7-hydroxycoumarin (umbelliferone). Malic acid itself condenses with orcinol in the presence of hot sulfuric acid to yield 7-hydroxy-5-methylcoumarin (homoumbelliferone).[2,3] This procedure is not applicable to α-substituted malic acids because they are degraded extensively in hot acid. The use of cold concentrated sulfuric acid limits the degradation of α-substituted malic acids to β-keto acids. Acetoacetic acid, the simplest β-keto acid, condenses with resorcinol in the presence of acid at room temperature to form 7-hydroxycoumarin.[4] Other β-keto acids undergo analogous condensations to yield fluorescent products. Hence, β-keto acids and compounds that are converted easily to β-keto acids, such as α-substituted malic acids, can be assayed fluorometrically after condensation with resorcinol.[5] Malic acids monosubstituted in the β-position may be similarly assayed but are considerably less fluorogenic.

Reagents

Conc. H_2SO_4, analytical grade, 96.1%

Conc. HCl, analytical grade

Resorcinol solution. Dissolve 1 g of resorcinol in 10 ml of H_2O just before use.

Sodium carbonate solution. Dissolve 28 g of anhydrous sodium carbonate in 100 ml of H_2O.

Borate buffer. Dissolve 7.32 g of boric acid in 100 ml of the sodium carbonate solution, dilute to 900 ml, adjust to pH 10 with 50% NaOH, and then dilute to 1 liter

Standards. Stock solutions of substituted malic acids are prepared by dissolving 1 mg of compound in 10 ml of ether at 0°

Water, redistilled 3 times from a glass still

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Special Apparatus

Fluorometer (G. K. Turner Associates, Palo Alto, California), model 110 null-balancing filter fluorometer. Primary filter No. 110-811 [7-60 (365 mμ)]; a secondary filter No. 110-817 [8 (485 mμ sharp cut)]; plus a No. 110-823 [N D 1% (100-fold reduction)]; and a 110-851 far UV lamp with the range selector (intensity) set at 1× for α-substituted malic acids, 10× for β-substituted malic acids, and 30× for malic acid

Cuvettes are round, quartz, and nonfluorescent

Procedure.[5] A reaction mixture may be freed of proteins by addition of a precipitant which is not soluble in ether (such as tungstic acid), and sufficient sulfuric acid to bring the mixture to pH 1–2. The solutions are clarified by filtration or centrifugation and extracted with ether in a continuous liquid-liquid extractor for 20 hours. The ether extracts are evaporated to dryness, and the residues are dissolved in 2 ml of anhydrous ether. Ether solutions are pipetted and aliquoted at 0°. Aliquots (0.1–0.2 ml) of the extracts and of the appropriate standard solutions are pipetted into 15 ml tapered glass-stoppered centrifuge tubes. The blank tube receives no sample. The solutions are evaported in a hood in a warm water bath at 32°, and completely dried at 65°. The tubes are cooled to room temperature, and 0.6 ml of conc. H_2SO_4 is added to each; they are stoppered, and mechanically shaken for 30 minutes. Then 0.5 ml of resorcinol solution is added, the tubes are shaken briefly, 1 ml of conc. HCl is added, and the tubes are shaken again. The tubes are stoppered and placed in the dark for 18–20 hours.

The contents of the tubes are transferred to 30 ml glass-stoppered round-bottom tubes. To make the transfer quantitative the small tubes are rinsed with 3 portions of 1 ml each of sodium carbonate solution, and these are added gradually to the larger tubes with shaking. The addition must be made slowly because vigorous effervescence occurs. Sufficient sodium carbonate solution is added to bring the solutions to pH 7.6 (a total of 7–8 ml). Borate buffer is added to bring the total volume to 12.5 ml. The final solution is at pH 8.5–9.0. Solutions are shaken and read in the fluorometer from immediately after preparation to within an hour thereafter.

Remarks. Fluorescence is directly proportional to concentration, with no evidence of quenching from 1 to 100 millimicromoles of α-substituted malic acids. Fluorescence is also dependent on the amount of sulfuric acid, with maximum fluorescence at 0.6 ml of sulfuric acid for 30 minutes. α-Substituted malic acids such as α-ethyl malic, α-methyl malic, α-isopropylmalic and citric acids show a much higher degree of fluores-

cence than β-substituted malic acids such as β-methyl malic and β-ethyl malic acid. Malic acid shows a still lower degree of fluorescence. The relationship of fluorescence to concentration is linear for all these compounds. α-Ketoisovaleric, α-ketoglutaric, succinic, homocitric, and oxaloacetic acid give no evidence of fluorescence.

[71] The Determination of Specific Radioactivities of Citric Acid Cycle Intermediates by Enzymatic Decarboxylation

By F. A. McElroy and G. R. Williams

For the effective use of labeled precursors in studies of the citric acid cycle, accurate determinations of the specific radioactivities of the cycle intermediates are required. Sensitive fluorimetric methods are available for the estimation of the total amount of the individual intermediates present in a mixture (see also this volume [65]). Several procedures involving an initial separation of the various intermediates have previously been employed to determine the total radioactivity content of each component. For example, Von Korff[1] has used the butanol-chloroform–silica gel system of Bulen, Varner, and Burrell,[2] Stuart and Williams[3] have used the paper chromatographic procedure of Lugg and Overell,[4] and Walter, Paetkau, and Lardy[5] have employed electrophoresis to separate the cycle intermediates. These methods are frequently time-consuming, require considerable quantities of starting material and, in addition, give no information concerning the precise location of the radioactivity in the compounds under consideration. An interesting approach to the latter problem has been taken by Bidwell,[6] who used the ninhydrin reaction to estimate the radioactivity content of the carboxylic group of amino acids. Enzymes have been used to a limited extent in studies of the location of labeling in dicarboxylic acids.[5,7]

In the present paper a method is described which takes advantage of the specificity of enzyme reactions to determine accurately the radioactivity content of specific positions in the intermediates of the citric acid cycle. Not only is this method rapid and highly sensitive, but it

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Acta Medica Univ. Kagoshima., Vol. 4, January 1962

Influences of Malic Acid on the Tissue Respiration of Rabbit Kidney Cortex

Yasuhisa Matumoto*, Yutaka Tokumitu* and Zyuniti Ukari*

In prveious papers were reported the influences of several saturated dibasic fatty acids on the tissue respiration of several organs of rabbits. Malic acid is one of the substances of TCA-cycle and has the important meaning to the metabolism of living-cells or -tissues and relates to fumaric acid or oxaloacetic acid. Influences of malic acid on the tissue respiration of lung, liver and cerebral cortex were reported by Nisimuta[1], Turuta[3] and Taniyama[2] in this laboratory. According to these reports, the substancial increase in the rate of oxygen consumption was observed. And so, in this paper will be reported the influences of malic acid on the tissue respiration of rabbit kindey cortex.

Methods

The oxygen consumption of tissue slices of rabbit kidney cortex was measured by Warburg's[4] old method. In these experiments was used the Phosphate-Ringer-Matumoto[5] as the suspension fluid. After 20 minutes preliminary experiment, every 30 minutes reading of each manometer was observed and the oxygen consumption was calculated from the change of manometric pressure as Qo_2 (per dry weight mg, per hour) and were showed the total two hours Qo_2s as TQo_2. The pH of suspension fluid of every vessel was measured before or after the experiment. From the changes of pH suspension fluid was calculated the change of the hydrogen ion concentration. Malic acid was added to the suspension fluid by the concentration of 5, 10 or 20 mg%.

Table 1. Qo_2 of kidney cortex slices

10 Samples	Qo_2				TQ_2	dry wt.	pH	
	I	II	III	IV			before	after
Cont.	7.01	7.13	7.68	7.27	29.09	4.8	7.37	7.05
±	0.13	0.22	0.18	0.22			0.01	0.01
10 mg%	7.82	8.08	8.42	7.68	32.00	5.4	7.09	6.99
±	0.16	0.20	0.24	0.22			0.01	0.01

Mean values ± probable error of the mean are presented.

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Results

The results of experiments in case of containing 10 mg% malic acid were shown in Table 1 to 3 and Fig. 1.

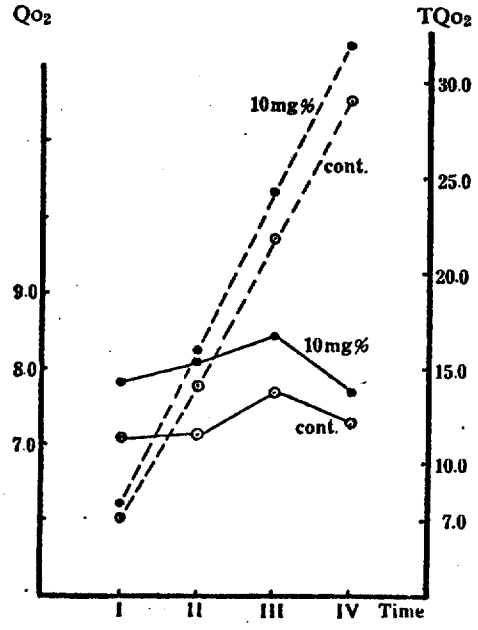
Table 2. TQo_2 of kidney cortex slices

	I	II	III	IV	Exp./Cont.
Cont.	7.01	14.14	21.82	29.09	1.00
10 mg%	7.82	15.90	24.32	32.00	1.10

Table 3. Changes of pH and hydrogen ion concentration of suspension fluid after the experiment

	before		after		diff.		dry wt.	per mg dry wt.	
	pH	$[H^+]\times10^{-8}$	pH	$[H^+]\times10^{-8}$	pH	$[H^+]\times10^{-8}$		$pH\times10^{-2}$	$[H^+]\times10^{-8}$
Cont.	7.37	4.27	7.05	8.91	0.32	4.64	4.8	6.67	0.97
10 mg%	7.09	8.13	6.99	10.20	0.10	2.07	5.4	1.85	0.38

Fig. 1. Oxygen consumption of kidney cortex slices of rabbit in case of containing 10 mg% malic acid



The results of experiments in case of containing 5 and 20 mg% malic acid were shown in Table 4 to 6 and Fig. 2.

Table 4. Qo_2 of kidney cortex slices

10 Samples	Qo_2				TQo_2	dry wt.	pH	
	I	II	III	IV			before	after
Cont.	6.58	6.62	6.77	6.40	26.38	6.6	7.34	6.96
±	0.10	0.24	0.15	0.07			0.01	0.01
5 mg%	7.22	6.97	7.28	6.71	28.18	6.7	7.23	6.97
±	0.18	0.22	0.14	0.09			0.01	0.01
20 mg%	7.39	7.33	7.80	7.23	29.75	6.8	6.81	6.96
±	0.14	0.22	0.25	0.10			0.01	0.02

Mean values ± probable error of the mean are presented.

Table 5. TQo_2 of kidney cortex slices

	I	II	III	IV	Exp./Cont.
Cont.	6.58	13.20	19.98	26.38	1.00
5 mg%	7.22	14.19	21.47	28.18	1.07
20 mg%	7.39	14.72	22.52	29.75	1.13

Table 6. Changes of pH and hydrogen ion concentration of suspension fluid after the experiment

	before		after		diff.		dry wt.	per mg dry wt.	
	pH	$[H^+]\times10^{-8}$	pH	$[H^+]\times10^{-8}$	pH	$[H^+]\times10^{-8}$		$pH\times10^{-2}$	$[H^+]\times10^{-8}$
Cont.	7.34	4.57	6.96	11.00	0.38	6.43	6.6	5.76	0.97
5 mg%	7.23	5.89	6.97	10.70	0.26	4.81	6.7	3.88	0.72
20 mg%	6.81	15.50	6.96	11.00	+0.15	+4.50	6.8	+2.21	+0.66

Discussion

Fumaric acid changes to malic acid by fumarase and malic acid to oxaloacetic acid by malic dehydrogenase. These substances and enzymes are the important members of TCA-cycle[6]. The oxygen consumption of tissues of living organs is related to TCA-cycle, generally and so malic acid has the important meaning to the tissue respiration.

According to the results of these experiments, malic acid increased the oxygen consumption of kidney cortex slices in proportion to the concentration within the range of 5 an 20 mg%. The pH of the suspension fluid before the experiment was small in proportion to the concentration of malic acid. The change of pH or hydrogen ion concentration was small in proportion to the concentration of malic acid, too. Especially, the pH of the suspension fluid became larger after the experiment than that before the experiment in case of 20 mg% of malic acid. In this laboratory were observed formerly the same results in kidney cortex slices in case of adding succinic acid or aspartic acid by Hasimoto[7] and Wada.[8] But these tendencies were not observed in the tissue slices of other

Flg. 2. Oxygen consumption of kidney cortex slices of rabbit in case of containing 5 and 20 mg% malic acid

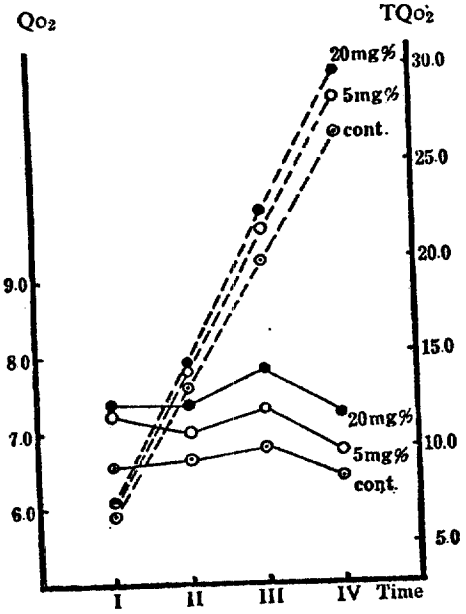
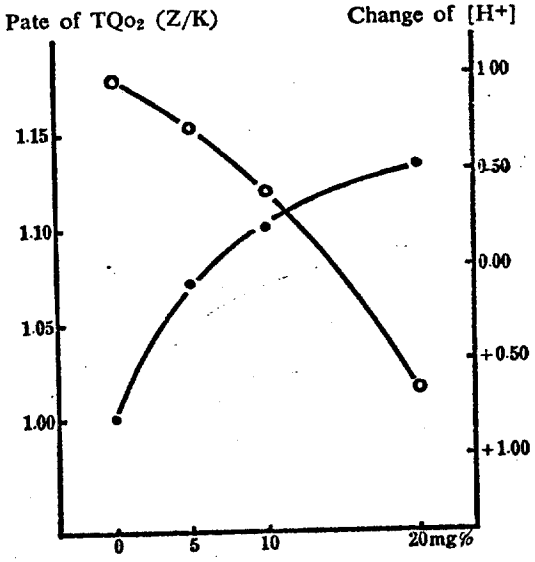


Fig. 3. The effect of the concentration of malic acid to the oxygen consumption and the change of hydrogen ion concentration before and after the experiment



organs. From these results were guessed the buffer actions of kidney cortex slices, in vitro, too (Fig. 3).

Summary

Influences of malic acid on the tissue respiration of rabbit kidney cortex were investigated. Obtained results were as follows.

1) Malic acid increased the oxygen consumption within the range of 5 and 20 mg% concentration. The increasing of the oxygen consumption was stronger in case of higher concentration of malic acid.

2) The change of pH or hydrogen ion concentration was small in proportion to the concentration of malic acid and the pH of the suspension fluid after the experiment, in case of containing 20 mg% malic acid was larger than that before the experiment, consequently the hydrogen ion concentration of the suspension fluid after the experiment was smaller as compared with that before the experiment.

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Generation of Extramitochondrial Reducing Power in Gluconeogenesis

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1. Kidney-cortex slices incubated with pyruvate formed glucose and lactate in relatively large and approximately equimolar quantities. The formation of these products involves two exclusively cytoplasmic $NADH_2$-requiring reductions, catalysed by lactate dehydrogenase and triose phosphate dehydrogenase. From the rates of glucose and lactate formation it can be calculated that over 1000 μ-moles of $NADH_2$ must have been produced in the cytoplasm/g. dry wt. of tissue/hr. 2. When lactate is a gluconeogenic precursor the required $NADH_2$ is generated in the cytoplasm, but, when a substrate more highly oxidized than glucose, such as pyruvate, is the precursor, there is no direct cytoplasmic source of $NADH_2$. Quantitative data on the fate of pyruvate are in accord with the conclusion that the $NADH_2$ was primarily formed intramitochondrially by the dehydrogenases of cell respiration, with pyruvate as the major substrate. 3. Similar observations and conclusions apply to experiments with mouse-liver slices incubated with pyruvate, serine or aspartate. 4. Addition of ethanol, which increases the formation of $NADH_2$ in the cytoplasm, increased the formation from pyruvate of lactate but not of glucose. 5. In view of the low permeability of mitochondria for NAD and $NADH_2$ it must be postulated that special carrier mechanisms transfer the reducing equivalents of intramitochondrially generated $NADH_2$ to the cytoplasm. Reasons are given in support of the assumption that the malate–oxaloacetate system acts as the carrier. 6. Various aspects of the generation of reducing power and its transfer from mitochondria to cytoplasm are discussed.

Gluconeogenesis from all amino acids, lactate, pyruvate and several other precursors involves a reductive step, namely the formation of glyceraldehyde phosphate from diphosphoglycerate, a reaction that occurs exclusively in the cytoplasm. This raises the question of the origin of the required reducing agent, $NADH_2$. When lactate is the gluconeogenic precursor, an obvious source of $NADH_2$ is the reaction lactate → pyruvate, which occurs in the cytoplasm in exact stoicheiometry with the reaction diphosphoglycerate → glyceraldehyde phosphate.

However, the origin of the $NADH_2$ is not obvious when added pyruvate acts as gluconeogenic precursor, because its conversion into glucose does not involve a cytoplasmic NAD-linked dehydrogenation: Evidence presented in this paper indicates that in this situation $NADH_2$ is primarily formed by the intramitochondrial dehydrogenases of the tricarboxylic acid cycle and connected reactions. Since the internal mitochondrial membrane is virtually impermeable to $NADH_2$, the transfer of $NADH_2$ to the cytoplasm requires a special carrier mechanism: $NADH_2$ reduces intramitochondrial oxaloacetate to malate, which diffuses into the cytoplasm and through the cytoplasmic malate dehydrogenase generates extramitochondrial $NADH_2$. This mechanism of generating $NADH_2$ in the cytoplasm must also operate for other precursors that are more highly oxidized than glucose, such as serine and glycerate.

EXPERIMENTAL

Methods. Slices of rat kidney cortex and mouse liver were incubated as described by Krebs, Bennett, de Gasquet, Gascoyne & Yoshida (1963*a*) and Krebs, Notton & Hems (1966). The phosphate-buffered saline of Krebs, Hems & Gascoyne (1963*b*) was used, with NaOH in the centre well of the manometer cups and O_2 in the gas space so that the O_2 consumption could be measured accurately. The methods used for the determination of metabolites were the same as in previous investigations in this Laboratory (Krebs, Dierks & Gascoyne, 1964; Gevers & Krebs, 1966; Hems, Ross, Berry & Krebs, 1966).

RESULTS

Products of pyruvate metabolism in rat kidney cortex. When pyruvate was added aerobically to slices of rat kidney cortex almost 1 mol. of pyruvate was removed/mol. of oxygen taken up (Table 1). The main products, apart from carbon dioxide, were glucose and lactate, which appeared in approximately equal molecular quantities. Small amounts of malate, fumarate, glutamate, glutamine, α-glycerophosphate and ketone bodies were also formed.

The reducing equivalents required for the formation of glucose and lactate from pyruvate (2 mol.

Table 1. *Fate of pyruvate and lactate in rat kidney-cortex slices*

Washed slices were incubated for 60 min. at 40° in 4 ml. of the phosphate-buffered saline of Krebs *et al.* (1963*b*). The gas space contained O_2. The animals had been starved for 24 hr. Disappearance of the metabolite is indicated by the − sign, formation by the + sign. All results in this and the other Tables represent averages of duplicates of single experiments.

	Metabolic changes (μmoles/g. dry wt./hr.)		
Substrate added	None	Pyruvate (10 mM)	L-Lactate (10 mM)
Metabolite			
O_2	−1060	−2130	−1865
Glucose	+ 19	+ 369	+ 197
Pyruvate	+ 2	−2020	+ 120
Lactate	+ 40	+ 334	− 736
Malate + fumarate	+ 1	+ 5	+ 2
Glutamate + glutamine	+ 8	+ 16	+ 23
α-Glycerophosphate	+ 1	+ 5	+ 2
Acetoacetate	+ 6	+ 16	
β-Hydroxybutyrate	+ 1	+ 4	

Table 2. *Analysis of data of Table* 1

It is assumed that the formation of malate and fumarate requires 1 mol. of pyruvate or lactate/mol., and the formation of glutamate, glutamine and ketone bodies 2 mol./mol.; it is further assumed that pyruvate yields 4 mol. of $NADH_2$/mol. and 1 mol. of reduced flavoprotein/mol. when undergoing degradation without electron transfer by the respiratory chain. No corrections were made for the small blanks observed in the control to which no substrate had been added, because the justification of any corrections is doubtful. The oxidations and reductions involved in the formation of glutamate, ketone bodies, lactate and pyruvate have been neglected.

Substrate added	Pyruvate	Lactate
Substrate removed accounted for as:		
Glucose (μmoles/g. dry wt.)	739	394
Lactate (μmoles/g. dry wt.)	334	—
Pyruvate (μmoles/g. dry wt.)	—	120
Malate + fumarate (μmoles/g. dry wt.)	5	2
Glutamate + glutamine (μmoles/g. dry wt.)	32	46
Ketone bodies (μmoles/g. dry wt.)	40	—
Total (μmoles/g. dry wt.)	1150	562
Pyruvate required for supply of $NADH_2$ (μmoles/g. dry wt.)	268	—
Substrate removed not accounted for (μmoles/g. dry wt.)	602	174
O_2 required for:		
(*a*) oxidation of substrate not accounted for (μmoles/g. dry wt.)	1505	522
(*b*) oxidation of reduced flavoprotein formed during the generation of $NADH_2$ (μmoles/g. dry wt.)	134	—
Percentage of total O_2 uptake accounted for by added substrate	77	28

of $NADH_2$/mol. of glucose, 1 mol. of $NADH_2$/mol. of lactate) equalled 1072 μmoles/g. dry wt. (uncorrected for the small changes in the control cup). No such amounts of hydrogen donors were available from preformed cell constituents because the concentrations of the substrates of NAD-linked dehydrogenases in the tissue were low; moreover, they decreased rather than increased on incubation with pyruvate. These quantitative considerations lead necessarily to the conclusion that the required $NADH_2$ was formed as the result of cell respiration, which in the experiment under discussion was mainly an oxidation of pyruvate, as the analysis of the data of Table 1 indicates (see Table 2). Much more pyruvate was removed than can be accounted for by the formation of glucose, lactate and the other products. These required 1150 μmoles of pyruvate/g. dry wt., whereas 2020 μmoles/g. dry wt. were removed. The excess, 870 μmoles/g. dry wt., is more than sufficient to allow the assumption that pyruvate was used to supply $NADH_2$ according to the reaction:

$$\text{Pyruvate} + 4\,\text{NAD} + \text{flavoprotein (FP)} + 3\,H_2O \rightarrow 3\,CO_2 + 4\,NADH_2 + FPH_2$$

To obtain 1072 μmoles of reducing equivalents/g. dry wt. by this reaction 268 μmoles of pyruvate/g. dry wt. are required, leaving 602 μmoles of pyruvate/g. dry wt. not accounted for. This fraction must have served as a substrate of respiration and was sufficient to contribute 77% of the respiratory fuel. More pyruvate was oxidized than can be accounted for by the extra oxygen uptake due to added substrate; thus pyruvate replaced a major part of the endogenous substrates of respiration.

When lactate was the added substrate there was also more lactate removed than could be accounted for by the products, but in this case the extra substrate used supplied only 28% of the total fuel for respiration and this was not sufficient for the extra oxygen consumption caused by the addition of lactate. Thus some of the extra respiration was due to an increased utilization of endogenous substrates.

The extra oxygen uptake caused by the addition of pyruvate or lactate was rather greater than might be expected on the basis of extra ATP requirements of gluconeogenesis, namely 6 mol. of ATP/mol. of glucose. With pyruvate as the substrate the extra ATP requirements were $369 \times 6 = 2214$ μmoles/g. dry wt., corresponding to an extra oxygen uptake of about 400 μmoles/g. dry wt., depending on the efficiency of oxidative phosphorylation. The observed extra oxygen uptake was 1070 μmoles/g. dry wt. With lactate as the substrate the expected extra oxygen uptake was about 200 μmoles/g. dry wt. and the observed value 805 μmoles/g. dry wt.

Products of pyruvate metabolism in mouse liver. When mouse liver was incubated with pyruvate analogous results were obtained (Table 3). Again much lactate was formed in addition to carbohydrate, and further $NADH_2$ was required for the formation of malate, fumarate and β-hydroxybutyrate. The analysis of the results is not as simple as for the kidney cortex because the changes in the control without pyruvate were much greater than in the kidney. In the latter tissue, in contrast with liver, the calculations are not much affected by deducting the control values. The sum of the metabolites formed in the presence of pyruvate in the liver was greater than the amount of pyruvate removed, which indicates that addition of pyruvate did not suppress the basic metabolic activities, as

Table 3. *Fate of pyruvate in mouse liver*

The general experimental conditions were as described in Table 1. To deplete the liver of glycogen, the mouse was treated with phlorrhizin (see Krebs *et al.* 1966) and left without food for 3 hr. Slices were cut dry and not washed. Analyses were carried out on slices plus medium.

Metabolite	Initial values (μmoles/g. dry wt.)	Values after 60 min. incubation (μmoles/g. dry wt.) Without added substrate	With pyruvate (10 mM)
O_2 used	—	355	532
Glucose + glycogen found	0·7	21	72
Pyruvate removed	—	—	456
Lactate found	7	27	131
Malate + fumarate found	0·3	1·6	42
Glutamate + glutamine found	44	7·7	5·6
Acetoacetate found	12	55	49
β-Hydroxybutyrate found	9	17	66
Total ketone bodies found	21	72	115
Acetoacetate/β-hydroxybutyrate ratio	1·33	3·2	0·74

it did to a large extent in kidney cortex. The increments over the control, in terms of $NADH_2$ requirements and expressed per g. dry wt., were 102 μmoles for glucose, 10$\frac{1}{2}$ μmoles for lactate, 40 μmoles for malate plus fumarate and 49 μmoles for β-hydroxybutyrate, a total of 295 μmoles/g. dry wt. The findings are in agreement with the postulate that the $NADH_2$ was formed as a result of mitochondrial dehydrogenase activity. The theoretical extra oxygen uptake due to ATP requirements was, as in the kidney experiments, much exceeded. A noteworthy observation is the fall of the acetoacetate/β-hydroxybutyrate ratio caused by pyruvate. It should be mentioned that the phosphate-buffered saline used to facilitate the measurement of the oxygen uptake was not the optimum medium for gluconeogenesis because of its low bicarbonate and carbon dioxide concentrations. For this reason the rate of glucose formation from pyruvate was lower than that reported by Krebs *et al.* (1966).

Metabolism of aspartate and serine. In principle the fate of serine in mouse liver (Table 4) and of L-aspartate in rat kidney cortex (Table 5) shows the same characteristics as that of pyruvate, though on a smaller scale because the rates at which these two substrates are metabolized are lower than those of pyruvate. Both carbohydrate and lactate were formed and the oxygen uptake was increased, particularly with aspartate. Since the degradation of serine and aspartate is not accompanied by cytoplasmic dehydrogenations, the reducing equivalents required for the formation of glucose and lactate must have arisen from intramitochondrial dehydrogenases.

Effect of ethanol. It was thought that the supply of extramitochondrial reducing equivalents is a factor limiting the rate of gluconeogenesis from pyruvate. Ethanol was added to increase the rate of formation of extramitochondrial $NADH_2$, because liver alcohol dehydrogenase is a cytoplasmic enzyme. In fact, there was a striking increase (more than doubling) of the lactate formation from pyruvate (Table 6), but the formation of glucose was not affected. Thus the additional $NADH_2$ reacted preferentially to reduce pyruvate rather than diphosphoglycerate.

Analogous observations were made in experiments on pigeon-liver homogenates (Table 7) in which ethanol together with crystalline liver

Table 4. *Fate of* L-*serine in mouse liver*

The general experimental conditions were as described in Table 1 except that pyruvate was replaced by L-serine.

Metabolite	Initial values (μmoles/g. dry wt.)	Values after 60 min. incubation (μmoles/g. dry wt.)	
		Without added substrate	With L-serine (10 mM)
O_2 used	—	216	344
Glucose + glycogen found	1	22	44
Pyruvate found	0·4	~0	15
Lactate found	7	20	39
Malate + fumarate found	1·3	4·8	5·1
Acetoacetate found	10	38	31
β-Hydroxybutyrate found	5·8	21	21
NH_3 + urea (as NH_3)	68	157	249

Table 5. *Fate of* L-*aspartate in slices of rat kidney cortex*

Washed slices were incubated in phosphate-buffered saline as described in Table 1. The rat had been fed on the casein–margarine diet specified by Krebs *et al.* (1963*a*).

Metabolite	Initial values (μmoles/g. dry wt.)	Values after 60 min. incubation (μmoles/g. dry wt.)	
		Without added substrate	With L-aspartate (10 mM)
O_2 used	—	936	2030
Glucose found	21	18	48
Pyruvate found	2	3	76
Lactate found	18	23	57
Malate + glutarate found	0·3	~0	12

Table 6. *Effect of ethanol on gluconeogenesis from pyruvate in mouse-liver slices*

The conditions of incubation and the treatment of the mouse were as described in Table 3.

		Values after 60min. incubation (μmoles/g. dry wt.)			
Metabolite	Initial values (μmoles/g. dry wt.)	Without added substrate	With ethanol (10mM)	With pyruvate (10mM)	With pyruvate (10mM)+ ethanol (10mM)
O_2 used	—	296	236	425	348
Glucose+glycogen found	0·4	25	17	57	60
Lactate found	7·3	15	34	92	198
Pyruvate used	—	—	—	343	398
Malate+fumarate found	—	—	—	4	4
Acetoacetate found	0·4	49	33	43	35
β-Hydroxybutyrate found	11·5	20	25	34	44

Table 7. *Effect of ethanol and alcohol dehydrogenase on the fate of pyruvate in pigeon-liver homogenate*

The homogenate was prepared as described by Krebs *et al.* (1964) from the liver of a starved (48hr.) pigeon. The final concentrations of added L-lactate and pyruvate were 40mM, of added NAD 0·5mM, of ethanol 20mM and of crystalline liver alcohol dehydrogenase (Dalziel, 1961) 0·25mg./ml. (0·003mM). Incubation was for 1hr. at 40°. The initial amounts of glucose+glycogen were 36μmoles/g. dry wt. and of lactate 20μmoles/g. dry wt. For general experimental details see Krebs *et al.* (1964).

	Values after 60min. incubation (μmoles/g. dry wt.)				
Substrate added	None	Ethanol	Lactate	Pyruvate	Pyruvate+ ethanol
Metabolite					
Glucose+glycogen found	1·4	1·3	239	49	41
Lactate found	0·3	16	—	232	1260

alcohol dehydrogenase was added (because the activity of alcohol dehydrogenase of pigeon liver is low). These additions increased the yield of lactate over fivefold without affecting the yield of glucose. The relatively low rate of glucose formation from pyruvate in pigeon-liver homogenate was thus not due to lack of $NADH_2$.

Experiments on perfused organs. Experiments on the isolated perfused rat liver and isolated perfused rat kidney in which pyruvate was added to the perfusion medium also indicated a substantial formation of lactate in addition to glucose. These experiments are not reported in detail in the present paper.

DISCUSSION

Appearance of cytoplasmic reducing power in the presence of pyruvate. The experiments show that large amounts of $NADH_2$, required for the conversion of diphosphoglycerate into glyceraldehyde phosphate and of pyruvate into lactate, are available in the cytoplasm when pyruvate is metabolized in kidney or liver slices. The location of these two reactions exclusively in the cytoplasm and the fact that they require $NADH_2$ as an obligatory reducing agent leave no doubt of this. The quantities (1072μmoles/g. dry wt./hr. in the experiments recorded in Table 1) are very high: they are of the same order as the basal oxygen uptake of kidney (1060μmoles/g. dry wt./hr.) and about 50% of the oxygen uptake in the presence of pyruvate. This means that in the presence of pyruvate about one-quarter of the total $NADH_2$ formed serves as a reducing agent and two-thirds as substrate of oxidative phosphorylation.

Since the permeability of mitochondria to the nicotinamide–adenine dinucleotides is very low (Lehninger, 1951; Kaufman & Kaplan, 1960; Purvis & Lowenstein, 1961), special mechanisms must be postulated for the supply of extramitochondrial $NADH_2$ in gluconeogenesis from substrates other than lactate, this being the only gluconeogenic precursor that forms $NADH_2$ in the cytoplasm in the required stoicheiometric proportions. Precursors more highly oxidized than lactate, such as pyruvate, glycerate, serine and oxaloacetate, generate no $NADH_2$ during their conversion into carbohydrate. Other precursors,

such as glutamate, aspartate, alanine, proline and ornithine, generate $NADH_2$, but solely in the mitochondria, since all the dehydrogenases involved in the conversion of these precursors into oxaloacetate are located intramitochondrially. This holds also for glutamate dehydrogenase, which is indirectly concerned with the degradation of many amino acids from which it arises by transamination.

There is an abundant supply of intramitochondrial $NADH_2$ in all respiring cells, formed by the dehydrogenases of the tricarboxylic acid cycle and the related reactions. But special carrier mechanisms must operate, transferring $NADH_2$ to the cytoplasm. How rapidly these mechanisms must operate is illustrated by the following calculations. The total $NADH_2$ content of rat kidney is less than 1·5 μmoles/g. dry wt. (Glock & McLean, 1955). Since 1072 μmoles of $NADH_2$/g. dry wt./hr. were used (Table 1), an amount equal to the total $NADH_2$ content of the liver was supplied, and must have traversed the mitochondrial membrane, every 5 sec.

Hydrogen carriers from mitochondria to cytoplasm. The postulated carrier systems must meet three requirements. They must readily accept hydrogen atoms from mitochondrial $NADH_2$. They must readily traverse the mitochondrial membrane. They must readily donate hydrogen atoms to extramitochondrial NAD. Thus they must be substances for which there are highly active NAD-linked dehydrogenases in both the intra- and extra-mitochondrial space. The only substrate couple that meets these three requirements in liver cells is the malate–oxaloacetate system. The isocitrate–oxalosuccinate system can be ruled out on account of the instability of oxalosuccinate and the virtual absence of an active NAD-linked isocitrate dehydrogenase in the cytoplasm. The conclusion is therefore inescapable that the malate–oxaloacetate system is the carrier converting intramitochondrial $NADH_2$ into extramitochondrial $NADH_2$. This conclusion is supported by entirely independent evidence obtained by Hoberman & D'Adamo (1960), who followed the fate of deuterium of 2-2′-deuterofumarate in starved rats. As expected, the deuterium appeared mainly in position 4 of glucose, with smaller quantities in position 6.

Physiological significance of the dicarboxylic acid shuttle. Evidence that malate is formed during gluconeogenesis from lactate and pyruvate was provided by the ^{14}C-labelling data of Topper & Hastings (1949) and of Lorber, Lifson, Wood, Sakami & Shreeve (1950). Their experiments with $^{14}CO_2$ and lactate labelled in position 2 or 3 indicated that at least 85% of C-2 and C-3 of lactate is randomized during the conversion into glucose, which indicates that it passes through the stage of fumarate. What had not been clear was the significance of this 'shuttle'. Earlier workers had assumed that it is an unavoidable side reaction of oxaloacetate due to the high activity of malate dehydrogenase. Later it was thought that 'malic' enzyme [malate dehydrogenase (decarboxylating) (NADP)] was the major catalyst in the formation of dicarboxylic acids from pyruvate (see Krebs, 1954), but this view had to be abandoned when evidence became available indicating that under physiological conditions 'malic' enzyme mainly reacts in the direction malate → pyruvate (Utter, Keech & Scrutton, 1964) and that its activity is relatively low under conditions of gluconeogenesis (Fitch & Chaikoff, 1960), and when it became clear that the formation of oxaloacetate is brought about by pyruvate carboxylase (Utter & Keech, 1960, 1963). The concept discussed in the present paper offers a satisfactory explanation for the fact that malate is formed during the gluconeogenesis from lactate: it is not formed by a useless 'shuttle' reaction but is an essential link in the transfer of hydrogen atoms to the glyceraldehyde phosphate–dehydrogenase system.

Sources of malate required as hydrogen carrier from mitochondria to cytoplasm. When substances more highly oxidized than glucose, such as pyruvate, are precursors the malate must be formed by the intramitochondrial reduction of oxaloacetate, the $NADH_2$ being supplied by the intramitochondrial dehydrogenases and their substrates, i.e. by the tricarboxylic acid cycle and the related reactions. When substances more reduced than glucose, such as glutamate, proline, propionate, citrate and succinate, are precursors, the malate is formed intramitochondrially during the oxidative degradation of the precursor and can diffuse into the cytoplasm. As mentioned above, no hydrogen transfer is required when lactate is the precursor. In this case oxaloacetate formed intramitochondrially from pyruvate may be transported to the cytoplasm either by direct diffusion or by the transamination mechanisms proposed by Lardy, Paetkau & Walter (1965).

Formation of lactate from pyruvate. In both kidney cortex and liver slices gluconeogenesis from an excess of added pyruvate was always accompanied by the formation of relatively large quantities of lactate. This is not surprising. Pyruvate is bound to compete with diphosphoglycerate for any $NADH_2$ available in the cytoplasm. Since the activity of lactate dehydrogenase is high, a formation of lactate, i.e. a diversion of the reducing power available in the cytoplasm from gluconeogenesis, is expected when an excess of pyruvate is present.

Source of reducing power for the formation of β-hydroxybutyrate from acetoacetate. β-Hydroxybutyrate dehydrogenase, being an intramito-

chondrial enzyme, requires intramitochondrial $NADH_2$ for the reduction of acetoacetate. The mechanism by which this $NADH_2$ is generated must be the same as in gluconeogenesis, i.e. by the intramitochondrial dehydrogenases of cell respiration, except that no transfer to the cytoplasm is required (Kulka, 1960; Kulka, Krebs & Eggleston, 1961; Krebs, Eggleston & D'Alessandro, 1961).

Role of energy-linked reduction of NAD in the generation of reducing power. It may be asked whether the energy-linked reduction of NAD, a well-established mitochondrial reaction, is of importance in the supply of $NADH_2$ for reductive syntheses. The following considerations show that the answer is essentially in the negative.

The energy-linked reduction of NAD involves a reversal of oxidative phosphorylation at the coupling stage between $NADH_2$ and flavoprotein, which may be formulated thus:

$$NADH_2 + FP + P_i \rightleftharpoons NAD + FPH_2 + \sim P \quad (1)$$

where FP represents an electron carrier, e.g. flavoprotein, and $\sim P$ an 'energy-rich' intermediate (or ATP). It may be left open whether FP and FPH_2 are in fact flavoproteins or electron carriers of a similar redox potential, such as ubiquinones. Reaction (1) implies that the energy-linked reduction of NAD, i.e. reaction (1) from right to left, depends on the stoicheiometric supply of $\sim P$ and FPH_2. Since $\sim P$ is plentiful in mitochondria the amount of FPH_2 is the major factor limiting the scope of the process. $NADH_2$, normally the most rapid supplier of FPH_2, cannot be a source, if reaction (1) proceeds from right to left. There remain as possible sources of FPH_2 the flavoprotein-linked dehydrogenases of succinate, fatty acyl-CoA esters, α-glycerophosphate, proline and choline. Of these substrates succinate is experimentally by far the most effective one because of the high activity of succinate dehydrogenase, and most of the experimental work demonstrating an energy-linked reduction has in fact been carried out in the presence of succinate (see Ernster & Lee, 1964). However, the amounts of succinate or acyl-CoA ester available *in vivo* are relatively small. Thus, when glucose is oxidized, one out of six dehydrogenations does not involve NAD but FP, and when fatty acids are oxidized the ratio is two out of six. Moreover, succinate and acyl-CoA esters must not only supply FPH_2, but also $\sim P$. Hence some of the FPH_2 must be oxidized by oxygen. Maximally 2 mol. of $\sim P$ could be formed/mol. of succinate or acyl-CoA, and only two-thirds of the succinate or acyl-CoA could take part in reaction (1). It follows that during carbohydrate oxidation one-ninth at most and during fatty acid oxidation two-ninths at most of the potential $NADH_2$ can be generated by energy-linked reduction. These are maximal values of what is theoretically feasible. The actual contribution of these systems to the production of $NADH_2$ is likely to be zero in animal tissues. The α-glycerophosphate oxidase cannot make a net contribution because the formation of α-glycerophosphate from triose phosphate requires the consumption of $NADH_2$.

The situation is entirely different in certain autotrophic micro-organisms, which can obtain all their energy from substrates the redox potentials of which are much more positive than those of the NAD couple, e.g. *Ferrobacillus* (Blaylock & Nason, 1963) or *Nitrosomonas* (Aleem, 1966). These organisms have an unlimited supply of substrates for oxidative phosphorylation at coupling sites between flavoproteins and oxygen. They can therefore generate bulk quantities of FPH_2 and $\sim P$. The organisms readily reduce NAD and it appears that an energy-linked reduction is the only mechanism available to them.

Even though the reversal of oxidative phosphorylation in animal tissues may not be an effective mechanism for generating $NADH_2$, it may nevertheless be useful to the economy of the cell by making possible a 100% utilization of energy.

Role of transamination in the transfer of oxaloacetate from mitochondria to cytoplasm. Lardy *et al.* (1965) and Haynes (1965) have discussed the concept of the diffusion of intramitochondrial malate into the cytoplasm with reference to the transfer of the carbon 'skeleton' of intramitochondrial oxaloacetate to the extramitochondrial space. They were not concerned with the transfer of reducing equivalent, a problem that arises with a limited number of glucogenic precursors of which pyruvate is the prototype. Lardy *et al.* (1965) also suggest that the intra- and extra-mitochondrial glutamate–oxaloacetate transaminase may play a role in transporting oxaloacetate from one compartment to another. Oxaloacetate is assumed to transaminate with glutamate in one compartment, and the products of transamination are postulated to diffuse into the other compartment where they react in the reverse direction. Such a mechanism can be visualized as transporting oxaloacetate from mitochondria to the cytoplasm without transporting reducing equivalents. It might therefore operate when carbon 'skeletons' but not reducing equivalents are to be transferred, as is the case when lactate is the precursor. A difficulty in accepting the hypothesis is the postulate that mitochondria are impermeable to oxaloacetate but readily permeable to α-oxoglutarate and aspartate. Evidence in support of the supposition of highly specific differential permeability is not entirely lacking but cannot be regarded as adequate (see Chappell & Haarhoff, 1966).

Cytoplasmic reducing power in fatty acid synthesis. When fatty acids are synthesized reducing equivalents have to be supplied in the cytoplasm in the form of $NADPH_2$. Most of this is generated by the pentose phosphate cycle and 'malic' enzyme (Young, Shrago & Lardy, 1964; Kornacker & Ball, 1965; Rognstad & Katz, 1966). These enzyme systems cannot play a major role in the generation of reducing power in gluconeogenesis because the activities of dehydrogenases of the pentose phosphate cycle and 'malic' enzyme are lower when gluconeogenesis is high and vice versa (Fitch & Chaikoff, 1960).

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PATHS OF CARBON IN GLUCONEOGENESIS AND LIPOGENESIS: THE ROLE OF MITOCHONDRIA IN SUPPLYING PRECURSORS OF PHOSPHOENOLPYRUVATE*

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Gluconeogenesis—the synthesis of glucose and glucose-containing polysaccharide from compounds other than hexoses—is a process of considerable magnitude in normal animals and one that is subject to grave alterations in certain disease states. In mammals gluconeogenesis occurs mainly, if not exclusively, in liver and kidney.[2] Carbohydrate is synthesized by these tissues from lactate and pyruvate during periods of heavy muscular work, and the glucose formed is returned to muscle to serve as a glycolytic energy source (Cori cycle). During long intervals between meals, and especially during fasting, amino acids from tissue proteins serve as a source of carbon for gluconeogenesis. In the absence of adrenal corticosteroids, protein reserves are not converted to carbohydrate sufficiently rapidly to maintain normal blood sugar levels[3] while in the diabetic this conversion is so rapid as to elevate blood sugar above the renal threshold.[4]

The main pathway of carbon in gluconeogenesis differs from the reverse of the glycolytic sequence at 3 steps[5] and consequently gluconeogenesis is subject to some controls that are without effect on carbohydrate degradation. One of these steps—the formation of phosphoenolpyruvate from pyruvate—seemed likely to be a site at which metabolic control of gluconeogenesis would be effected[6] since it is at the point where pyruvate from either lactate or amino acid residues enters the route to hexose formation.

Pyruvate enters the gluconeogenic route by being carboxylated to a dicarboxylic acid.[7] This is accomplished by the pyruvate carboxylase (reaction 1) of Utter and Keech which is located predominantly in the mitochondria of liver cells:[8–11]

$$\text{Pyruvate} + CO_2 + \text{ATP} \xrightarrow{\text{acetyl CoA}} \text{Oxalacetate} + \text{ADP} + P_i. \qquad (1)$$

Pyruvate carboxylase increases in amount in the livers of fasted rats,[13] those treated with hydrocortisone,[13] and diabetic rats[11, 12] in keeping with its proposed role in gluconeogenesis. Another possible means of carboxylating pyruvate—via malic enzyme[14]—was suggested by Wagle and Ashmore[15] to be involved in the enhanced gluconeogenesis of the diabetic rat. However, this enzyme is not sufficiently active to account for the pyruvate converted to carbohydrate in normal liver, is not induced by fasting or hydrocortisone, and is in fact greatly diminished in diabetic rats' livers.[6] Instead, malic enzyme participates in lipogenesis by converting oxalacetate, via malate, to pyruvate and generating TPNH.[16, 17]

Phosphoenolpyruvate carboxykinase, the enzyme that converts oxalacetate to phosphoenolpyruvate (reaction 2), was discovered by Utter and Kurahashi[18] and was found to be located in the soluble fraction of rat, mouse, and hamster liver.[19]

$$\text{Oxalacetate} + \text{GTP (or ITP)} \rightleftarrows \text{Phosphoenolpyruvate} + \text{GDP (or IDP)} \qquad (2)$$

Its activity in liver is greatly enhanced by fasting, by diabetes (induced by alloxan,

mannoheptulose or pancreatectomy) and allowing the administration of gluconeogenic precursors.[20] It is sufficiently active to account for the rate of gluconeogenesis in normal rat liver and in the metabolic alterations mentioned immediately above.[1]

These findings indicated that pyruvate must be carboxylated to oxaloacetate in the mitochondria, whereas conversion of oxaloacetate to phosphoenolpyruvate and the succeeding reactions of gluconeogenesis occur in the extramitochondrial portion of the cell.

Experiments designed to verify this scheme led to the finding that *virtually no oxaloacetate accumulated in media containing pyruvate, bicarbonate, and rat liver mitochondria* under a variety of incubation conditions (Table 1 and experiments to be presented elsewhere). The addition of creatine and crystalline creatine kinase[22] as a phosphate acceptor system to enhance pyruvate oxidation[23] did not enhance oxaloacetate production. Therefore, the production of other 4-carbon compounds by mitochondria was studied.

When malate and glutamate are both supplied to mitochondria (expts. 5–7), *oxaloacetate is produced and liberated to the soluble phase of the system at a rate of only 0.11 μmole per minute by mitochondria from 1 gm of liver.* This is about 10

TABLE 1

AN EXAMINATION OF THE METABOLIC PATHWAY FROM PYRUVATE TO PHOSPHOENOLPYRUVATE

Expt. no.	Source of intramitochondrial OAA and/or aspartate	Extramitochondrial OAA-trapping system	Rate of product accumulation*
1	7 mM Pyr, 10 mM bicarbonate	None	0.015
2	7 mM Pyr, 10 mM bicarbonate, Cr-Crk	None	0.005
3	7 mM Pyr, 10 mM bicarbonate, 5 mM Glu	None	0.045
4	7 mM Pyr, 10 mM bicarbonate, 5 mM Glu, Cr-Crk	None	0.021
5	5 mM malate, 5 mM Glu, Cr-Crk	MDH, 0.5 mM DPNH (1 mM αKG present)	0.11
6	5 mM malate, 5 mM Glu, Cr-Crk	MDH, 0.5 mM DPNH, GOT, 1 mM αKG	0.99
7	5 mM malate, 5 mM Glu	MDH, 0.5 mM DPNH, GOT, 1 mM αKG	0.54
8	7 mM Pyr, 10 mM bicarbonate, 3 mM Glu	None	0.00
9	7 mM Pyr, 10 mM bicarbonate	1.8 U PEP-CK	0.04
10	7 mM Pyr, 10 mM bicarbonate, 3 mM Glu	2.5 U PEP-CK	0.04
11	7 mM Pyr, 10 mM bicarbonate, 3 mM Glu	1.25 U PEP-CK, GOT	0.13
12	7 mM Pyr, 10 mM bicarbonate, 3 mM Glu	1.8 U PEP-CK, GOT	0.29
13	7 mM Pyr, 10 mM bicarbonate, 3 mM Glu	2.5 U PEP-CK, GOT	0.29
14	7 mM Pyr, 10 mM bicarbonate	0.75 U PEP-CK	0.00
15	7 mM Pyr, 10 mM bicarbonate	1.50 U PEP-CK	0.00
16	7 mM Pyr, 10 mM bicarbonate, 3 mM Glu	0.75 U PEP-CK	0.09
17	7 mM Pyr, 10 mM bicarbonate, 3 mM Glu	1.50 U PEP-CK	0.17
18	7 mM Pyr, 10 mM bicarbonate, 3 mM Glu	0.75 U PEP-CK, GOT	0.23
19	7 mM Pyr, 10 mM bicarbonate, 3 mM Glu	1.50 U PEP-CK, GOT	0.43
20	7 mM Pyr, 10 mM bicarbonate, 3 mM Glu (2 mM malonate present)	0.75 U PEP-CK, GOT	0.31
21	7 mM Pyr, 10 mM bicarbonate, 3 mM Glu (2 mM malonate present)	1.50 U PEP-CK, GOT	0.43

*μmoles/min/gm liver.

OAA = oxaloacetate; Glu = glutamate; Asp = aspartate; αKG = α-ketoglutarate; Pyr = pyruvate; Cr-Crk = 13 mM creatine + 0.1 mg crystalline creatine kinase per ml; MDH = 10 units of crystalline malate dehydrogenase (Boehringer); GOT = 10 units of glutamate-oxalacetate transaminase (Boehringer) dialyzed free of ammonia; PEP = phosphoenolpyruvate; PEP-CK = PEP carboxykinase.[18,24]

In all experiments, ATP = 3 mM; pH = 7.4; mitochondria from 0.17 gm rat liver were included in each ml of reaction mixture, except in expts. 5–7 where mitochondria from 6.6 mg of liver were used. In expts. 1–4, T = 37°; P_i = 6 mM; triethanolamine (Cl⁻) buffer = 7 mM; $MgSO_4$ = 6 mM; oxalacetate was determined by the highly sensitive colorimetric method of Kalnitsky and Tapley.[21] In expts. 5–7, T = 21°; P_i = 1 mM; triethanolamine (Cl⁻) buffer = 25 mM; $MgSO_4$ = 6 mM; DPNH oxidation was measured spectrophotometrically at 340 mμ. In expts. 8–21, T = 37°; P_i = 3 mM; triethanolamine (Cl⁻) buffer = 10 mM; $MgSO_4$ = 18 mM; UTP = 5 mM; PEP was determined chemically.[19] A control for expts. 5–7 had a zero rate of DPNH oxidation in the absence of either malate or malate dehydrogenase.

per cent of the rate required for normal gluconeogenesis.[7] The addition of glutamic-oxalacetic transaminase in the presence of 1 mM α-ketoglutarate resulted in DPNH oxidation at the rate of 1 μmole per minute. The data of these experiments indicate that malate was oxidized to oxalacetate in the mitochondria and transaminated there to form aspartate which diffused from the mitochondria. The added glutamate-oxalacetate transaminase and α-ketoglutarate convert aspartate to oxalacetate, and the latter oxidizes extramitochondrial DPNH in the presence of malate dehydrogenase. Omission of the phosphate acceptor system (expt. 7) slowed malate oxidation in the mitochondria to the point where only half as much aspartate was formed.

In the remaining experiments (8–21) of Table 1, pyruvate plus HCO_3^- was added to produce C_4 acids; enzyme systems for converting (extramitochondrially) either oxalacetate [(*i*), PEP-CK + ITP] or aspartate [(*ii*), GOT + PEP-CK + ITP] to phosphoenolpyruvate were added, and the latter compound was measured.[19] With system (*i*), or without glutamate, only negligible amounts of phosphopyruvate accumulated. Only in system (*ii*) was phosphopyruvate formation significant (expts. 18–21). In these experiments no ketoglutarate or malate was added. Oxalacetate formed by carboxylation of pyruvate transaminated with glutamate; the aspartate and ketoglutarate formed diffused out of the mitochondria where the added transaminase converted them partially to oxalacetate and glutamate. In similar ezperiments (to be published elsewhere) but without an external trapping system, about one μmole of aspartate and 0.73 μmoles of α-ketoglutarate were liberated per minute by the mitochondria from 1 gm of liver. Thus the availability of α-ketoglutarate may be limiting the rate of phosphopyruvate formation under these conditions. Considering that no attempts were made to determine conditions for maximum rates of phosphopyruvate production, the yields of the latter are reasonable.

TABLE 2

FORMATION OF ORGANIC ACIDS* FROM PYRUVATE, BICARBONATE, AND GLUTAMATE BY RAT LIVER MITOCHONDRIA

Acids	System without Glutamate 0–5 min	System without Glutamate 0–10 min	System with Glutamate 0–5 min	System with Glutamate 0–10 min
Pyruvate used	3.47	3.70	3.80	3.64
Glutamate used	—	—	1.30	1.13
Malate formed	0.87	0.85	1.09	1.05
C^{14}-Malate formed†	0.75	0.68	0.84	0.77
Citrate formed	0.68	0.72	0.52	0.56
C^{14}-Citrate formed†	0.56	0.56	0.35	0.42
Aspartate formed			0.85	0.90
C^{14}-Aspartate formed†			0.70	0.62
Alanine formed			0.40	0.28
α-Ketoglutarate formed	0.11	0.06	0.56	0.40
Total C^{14} products†	1.31	1.24	1.89	1.81

* μmoles/min/gm liver.

† Calculated on the basis of the specific radioactivity of the $KH^{14}CO_3$, assuming that not more than one CO_2 has been incorporated per molecule.

The reaction mixture contained 3 mM ATP, 7.5 mM $MgSO_4$, 6.7 mM potassium phosphate, pH 7.4, 6.7 mM triethanolamine, pH 7.4, 10 mM $KH^{14}CO_3$ (0.6 μc/μmole), 6.7 mM sodium pyruvate, 1.0 ml mitochondrial suspension corresponding to 0.8 gm of original rat liver, and, when indicated, 3 mM potassium glutamate. All components were added as essentially isotonic solutions, and the final volume was made up to 6.0 ml with 0.25 *M* sucrose. The incubations were carried out in stoppered 25-ml Erlenmeyer flasks which were shaken at 37° in a water bath. The reaction was started by adding the mitochondria after an equilibration period of 2 min and stopped by adding 6 ml of 0.66 *M* $HClO_4$. The deproteinized samples were neutralized with KOH, and the perchlorate salt was centrifuged off. An aliquot was freed of all nonacid components by chromatography on Dowex-2-formate, and the acids were then separated by high-voltage electrophoresis at 4500 v and pH 3 on Whatman 3 MM paper strips. The radioactive peaks were located on a paper scanner, cut out, and counted in the scintillation counter. The content of the various acids was determined according to the references cited: malate,[25] pyruvate,[25] α-ketoglutarate,[25] citrate;[26] the amino acids were determined on the Spinco amino acid analyzer.

The insensitivity of system (*iii*) to malonate suggests that glutamate is required as an amino group donor rather than as a carbon source for aspartate formation (expts. 20 and 21). This is confirmed by the isotopic experiments which follow.

In the experiments summarized in Table 2, the production of various organic acids from pyruvate and $H^{14}CO_3^-$ was measured and their specific radioactivity was determined. Malate and citrate were produced in good yields, while only a small amount of α-ketoglutarate and negligible amounts of isocitrate were found. The specific radioactivity of malate and citrate indicated that at least 78–86 per cent of the *oxalacetate* going into these compounds was derived directly from carboxylated pyruvate. These values are minimal because they are calculated from the specific activity of the $H^{14}CO_3^-$ added and do not take into account the dilution by metabolically produced CO_2. Any malate or oxalacetate originating from the tricarboxylic acid cycle would not be labeled. Thus, in mitochondria under these conditions, malate arises almost entirely by reduction of oxalacetate, and not by the tricarboxylic acid cycle.

In the presence of glutamate, malate formation was slightly enhanced and at least 73–77 per cent originated from oxalacetate formed by direct carboxylation of pyruvate. Aspartate was produced in amounts significant for gluconeogenesis and this compound too originated largely (at least 69–82%) from oxalacetate produced by pyruvate carboxylation. Glutamate diminished citrate formation slightly but enhanced total C^{14} fixed by trapping oxalacetate as aspartate.

When the phosphate acceptor system hexokinase and glucose was added to the mixtures described in Table 2, total C^{14} fixed in the system in the absence and presence of glutamate, respectively, decreased to 3 and 6 per cent of the amount fixed without phosphate acceptor.

Discussion.—The implications of these findings for gluconeogenesis are summarized in Figure 1. Both malate and aspartate are likely precursors of oxalacetate in the extramitochondrial compartment of the liver cell. The malate oxalace-

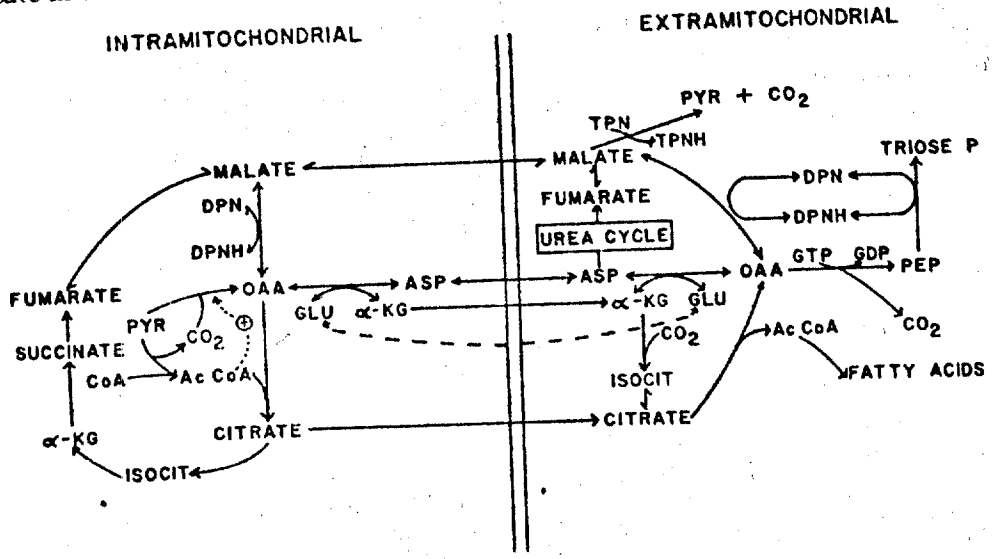

FIG. 1.

tate couple is in equilibrium with other pyridine nucleotide-linked systems in rat liver cytoplasm.[27] Thus, malate *can* form oxalacetate in this compartment, despite an unfavorable equilibrium constant, because of the existing high ratios of malate to oxalacetate and of DPN to DPNH. The DPNH continually formed during malate oxidation may serve for the reduction of 1,3-diphosphoglycerate to triose phosphate. Alternatively, malate can serve as a source of TPNH, via malic enzyme, for fat synthesis.[16, 17] Aspartate may be transaminated with α-ketoglutarate to produce oxalacetate for gluconeogenesis and glutamate which may again enter the mitochondria to transaminate with oxalacetate produced by pyruvate carboxylase. Aspartate is also the source of an amino group for urea synthesis[28] by a pathway which yields fumarate.

The reactions proposed provide a function for the glutamate-oxalacetate transaminases found in both the soluble and mitochondrial fractions of the liver cell.[29, 30] They provide a role for fumarase, malate dehydrogenase, and malic enzyme in the cytoplasm. In mitochondria, the observed rate of $H^{14}CO_3^-$ incorporation into the 4-carbon compounds, malate and aspartate, is sufficient to account for the rate of gluconeogenesis from pyruvate in liver, i.e., 1 μmole of pyruvate per minute, per gram of liver.[7] The enzymes of the soluble portion of rat liver homogenate catalyze PEP formation from added oxalacetate, aspartate, or malate considerably faster than the minimum required for gluconeogenesis.[31, 32]

The rates of $^{14}CO_2$ fixation observed in the present work are, to our knowledge, by far the greatest reported for intact mitochondria. For example, Freedman and Kohn[11] reported 0.16 μmole of CO_2 fixed per gram of liver in 5 min at 30°. Our rates, at 37°, are 60-fold higher. As will be reported in detail elsewhere, inorganic phosphate is required to achieve these rates of CO_2 fixation by intact mitochondria. Equally important is the absence of a phosphate acceptor which depletes the ATP reserves, as was pointed out above. In the experiments of Krebs, Dierks, and Gascoyne,[33] lactate was converted to carbohydrate at a rate of nearly 1 μmole per minute per gram of homogenized pigeon liver. Presumably, pyruvate was carboxylated at good rates in the mitochondria of their homogenates.

Summary.—It is concluded that gluconeogenesis in rat liver involves carboxylation of pyruvate to oxalacetate in mitochondria. Oxalacetate does not diffuse from the mitochondria but is transaminated to form aspartate or reduced to malate. Aspartate, malate, α-ketoglutarate, and some citrate diffuse from mitochondria. In the extramitochondrial compartment of the cell, oxidation of malate and transamination of aspartate yield oxalacetate which can be converted to phosphoenolpyruvate by the soluble carboxykinase. The production, in mitochondria, of a variety of compounds that may serve as precursors of phosphoenolpyruvate in the extramitochondrial compartment provides a multiplicity of sites for metabolic controls.

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*SURFACE FEATURES OF THE 50S RIBOSOMAL COMPONENT OF ESCHERICHIA COLI**

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The mechanism by which ribosomes function in protein synthesis will not be known until their molecular arrangement has been determined. Thus far it has been established that the *Escherichia coli* ribosome has two major structural subunits, sedimenting at 50S and 30S, each of which consists of a long polyribonucleotide chain loosely bonded to a number of globular protein molecules.[1–3] These molecular constituents interact to impose a coiling of the polynucleotide chain; thus, the assemblage is nearly symmetrical in its external dimensions,[4] but loose enough to permit a high degree of internal hydration.[5] The manner of folding of the polynucleotide chain and its spatial relationships with the protein molecules should be discernible by electron microscopy. To this end, the isolated 50S component has been examined in frozen-dried, tungsten-shadowed preparations, and